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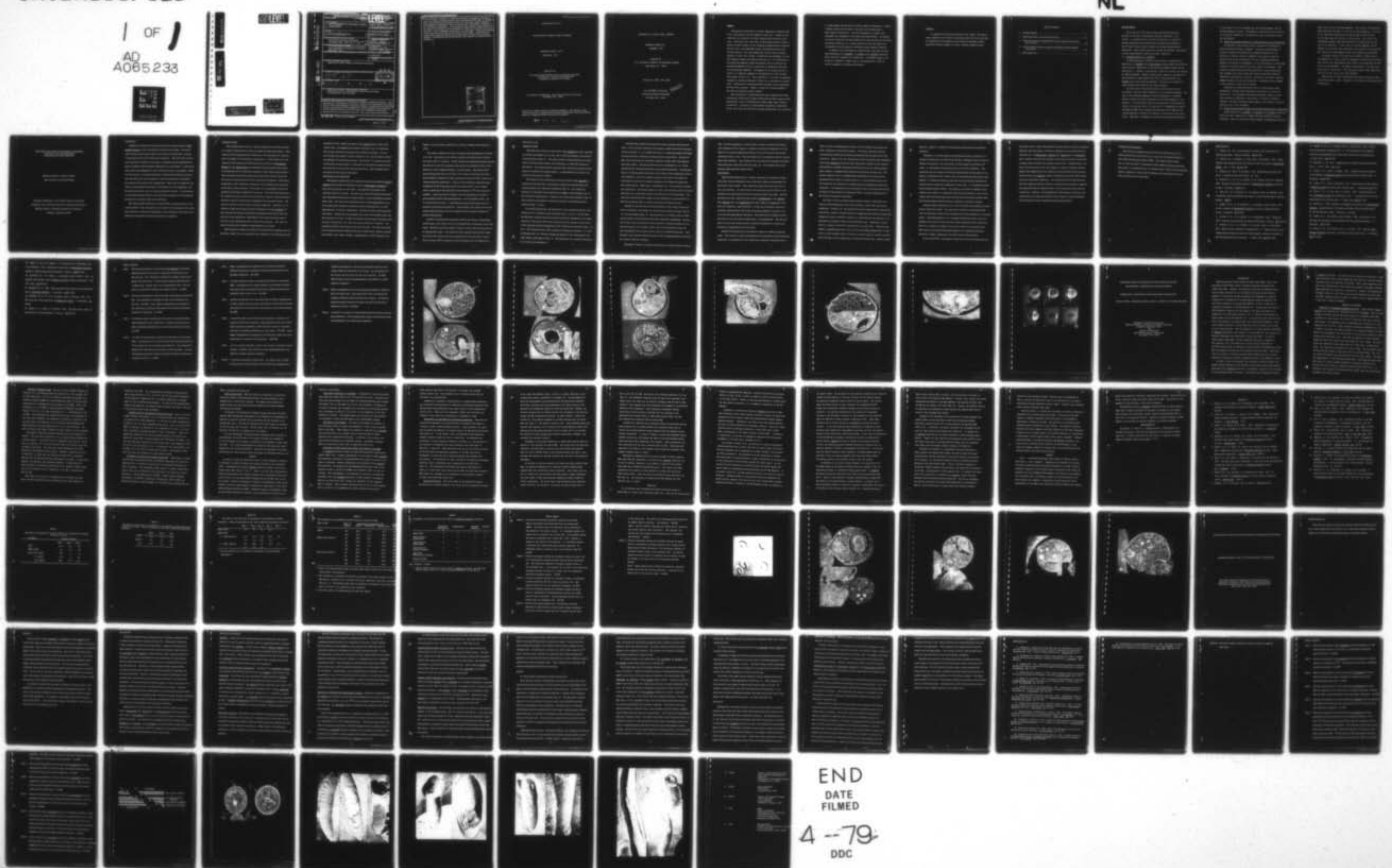

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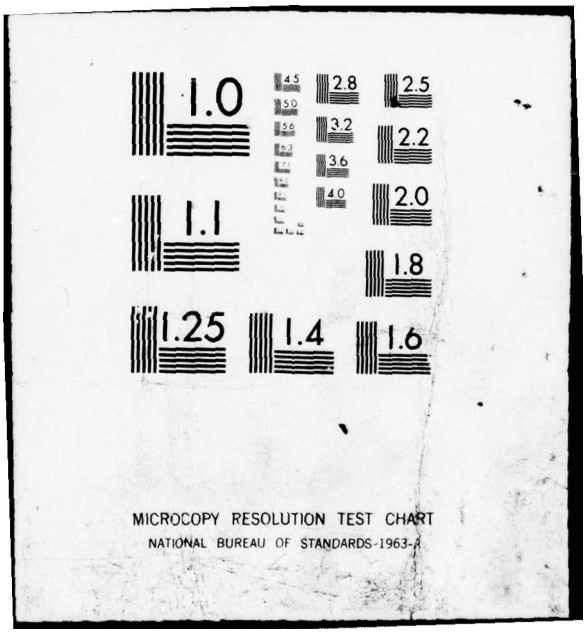
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INTRODUCTION

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Host-parasite interaction is of major importance to malarial parasites, since parasite survival depends on host cells. Studies on the interaction are essential for better understanding of malarial parasites. During the last fiscal year we have concentrated our investigation on several aspects of the interaction between malarial parasites and host cells, and established several new characteristics on the invasion process. This study showed that invasion

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occurs through a sequence of events that include: (1) recognition and attachment, (2) junction formation between the merozoite and host cell, (3) invagination of the erythrocyte membrane around the merozoite, and (4) sealing of the invaginated membrane to form a vacuole and to reestablish continuity of the erythrocyte membrane. The movement of the junction during invasion is an important component of the mechanism by which the merozoite enters the host cell. After completion of this work, we developed a method for blocking erythrocyte invasion by the merozoite at an early stage. Cytochalasin B-treated merozoites attached to host erythrocytes and then form a junction. However, cytochalasin blocked movement of the junction preventing further invasion.

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Cytochemistry of Malaria Under Treatment

Masamichi Aikawa, M.D.

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The Institute of Pathology, Case Western Reserve University
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Cytochemistry of malaria under treatment.

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Summary

Host-parasite interaction is of major importance to malarial parasites, since parasite survival depends on host cells. Studies on the interaction are essential for better understanding of malarial parasites. During the last fiscal year, we have concentrated our investigation on several aspects of the interaction between malarial parasites and host cells, and established several new characteristics on the invasion process. This study showed that invasion occurs through a sequence of events that include 1) recognition and attachment, 2) junction formation between the merozoite and host cell, 3) invagination of the erythrocyte membrane around the merozoite and 4) sealing of the invaginated membrane to form a vacuole and to reestablish continuity of the erythrocyte membrane. The movement of the junction during invasion is an important component of the mechanism by which the merozoite enters the host cell. After completion of this work, we developed a method for blocking erythrocyte invasion by the merozoite at an early stage. Cytochalasin B treated merozoites attached to host erythrocytes and then form a junction. However, cytochalasin blocked movement of the junction preventing further invasion.

When cytochalasin B treated merozoites were incubated with rhesus erythrocytes, Duffy positive human erythrocytes and Duffy negative human erythrocytes, certain differences were present among these different erythrocytes. A junction is found between cytochalasin treated merozoites, and rhesus and Duffy positive human erythrocytes, but no junction

is formed between the merozoites and Duffy negative erythrocytes. Instead, filaments extend from the edge of the apical end of merozoites to Duffy negative erythrocytes. From this observation, it appears that attachments are independent of the Duffy associated antigen. The absence of junction formation with Duffy negative cells may indicate that the Duffy associated antigen acts as a second receptor for junction formation or a determinant on Duffy negative erythrocytes blocks junction formation.

In addition, we investigated by freeze fracture techniques the membrane morphology of P. cynomolgi, P. knowlesi and P. berghei sporozoites before and after incubation with immune sera. A prominent change in the sporozoite incubated in immune serum is the appearance of a layer of particle aggregates surrounding the parasite.

Foreward

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Science - National Research Council.

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Detailed Report

During the last fiscal year, we have investigated three major problems in relation to host-malarial parasite interaction. They include: 1) erythrocyte entry by malarial parasites, 2) interaction between cytochalasin B-treated malarial parasites and erythrocytes, and 3) freeze fracture study on normal and antibody-treated malarial sporozoites. These studies resulted in several new findings, contributing a better understanding of malarial and host interaction.

(1) Erythrocyte entry by P. knowlesi.

In 1969 Ladda et al. reported on the invasion of erythrocytes by merozoites of P. berghei and P. gallinaceum and established that merozoites enter within an invagination of the erythrocyte membrane rather than by penetrating it. However, ultrastructural studies on invasion were hindered by sampling problems. Recently, Dennis et al. reported a new method for the collection of large quantities of free, viable merozoites of P. knowlesi which could invade erythrocytes. By applying this method, we report several new findings on the invasion process.

The apical end of the merozoite makes initial contact with the erythrocyte, creating a small depression in the erythrocyte membrane. The area of the erythrocyte membrane to which the merozoite is attached becomes thickened and forms a junction with the plasma membrane of the merozoite. As the merozoite enters the invagination in the erythrocyte surface, the junction, which is in the form of a circumferential zone of attachment between the erythrocyte and merozoite, moves along the confronted membranes to maintain its position at the orifice of the invagination. When entry is completed, the orifice closes behind the parasite

in the fashion of an iris diaphragm, and the junction becomes a part of the parasitophorous vacuole. The movement of the junction during invasion is an important component of the mechanism by which the merozoite enters the erythrocytes.

(2) Interaction between cytochalasin B-treated merozoites and erythrocytes.

As described in the previous section, invasion of erythrocytes by malarial merozoites follows a sequence: recognition and attachment in an apical orientation associated with widespread deformation of the erythrocyte, junction formation, movement of the junction around the merozoite that brings the merozoite into the invaginated erythrocyte membrane, and sealing of the membrane. In this investigation, we described a method for blocking invasion at an early stage in the sequence. Cytochalasin B-treated merozoites attach specifically to host erythrocytes, most frequently the apical region that contains specialized organelles (rhoptries) associated with invasion. The parasite then forms a junction between the apical end and the erythrocyte. Cytochalasin blocks movement of this junction, a later step in invasion.

Cytochalasin B-treated merozoites attach to Duffy negative human erythrocytes, although these erythrocytes are resistant to invasion by the parasite. The attachment with these erythrocytes, however, differs from susceptible erythrocytes in that there is no junction formation. Therefore, the Duffy associated antigen appears to be involved in junction formation, not initial attachment.

(3) Freeze fracture study on normal and antibody-treated malarial sporozoites.

Freeze fracture of P. cynomolgi, P. knowlesi and P. berghei sporozoites before and after incubation with immune serum were studied by electron microscopy. There are evenly distributed numerous intramembranous particles

(IMP) on the P face of the outer membrane. The E face of the plasma membrane shows fewer IMP than the P face of the plasma membrane. The E face of the intermediate membrane has few IMP and shows linear slightly raised ridges along the long axis of the parasite. The P face of the intermediate membrane shows many IMP which are aligned along the long axis of the sporozoite. On the P face of the inner membrane, IMP are aligned in very distinct rows conforming to the long axis of the parasite. The E face of the inner membrane shows a few randomly distributed IMP.

A prominent change in the sporozoite incubated in immune serum is the appearance of a layer of particle aggregates surrounding the parasite. The P face of the plasma membrane shows several clear areas devoid of IMP as well as IMP aggregates. No changes are detectable in the other fractured faces of the pellicle. This observation suggests that immune serum only acts on the P face of the plasma membrane.

Detailed data of these investigations have been submitted for publication and are now in press. Attached to this report are three manuscripts of these data.

ERYTHROCYTE ENTRY BY MALARIAL PARASITES: A MOVING JUNCTION BETWEEN ERYTHROCYTE AND PARASITE.

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James Johnson, and John Rabbege

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ABSTRACT

Invasion of erythrocytes by merozoites of the monkey malaria, Plasmodium knowlesi, was investigated by electron microscopy. The apical end of the merozoite makes initial contact with the erythrocyte, creating a small depression in the erythrocyte membrane. The area of the erythrocyte membrane to which the merozoite is attached becomes thickened and forms a junction with the plasma membrane of the merozoite. As the merozoite enters the invagination in the erythrocyte surface, the junction, which is in the form of a circumferential zone of attachment between the erythrocyte and merozoite, moves along the confronted membranes to maintain its position at the orifice of the invagination. When entry is completed, the orifice closes behind the parasite in the fashion of an iris diaphragm, and the junction becomes a part of the parasitophorous vacuole. The movement of the junction during invasion is an important component of the mechanism by which the merozoite enters the erythrocyte.

The extracellular merozoite is covered with a prominent surface coat. During invasion, this coat appears to be absent from the portion of the merozoite within the erythrocyte invagination, but the density of the surface coat outside the invagination (beyond the junction) is unaltered.

INTRODUCTION

The asexual malaria parasite infects erythrocytes and there develops to a mature schizont that is made up of many individual merozoites. Upon rupture of the schizont infected erythrocyte, the merozoites are released and are capable of infecting other erythrocytes. In 1969 Ladda et al. (15) reported on the invasion of erythrocytes by merozoites of Plasmodium berghei and P. gallinaceum and established that merozoites enter within an invagination of the erythrocyte membrane rather than by penetrating it. They found that merozoites approached erythrocytes with the apical end and formed a focal depression on the erythrocyte membrane. With deeper invagination of the erythrocyte membrane, the resulting cavity conformed to the shape of the merozoite. The orifice of the invaginated erythrocyte membrane fused upon completion of the entry. They also noted that granular material (a surface coat) covered the entire surface of the extracellular merozoite and was removed upon completion of the merozoite entry. The surface coat was at least in part of parasite origin (16). Dvořak et al. (7) studied by light microscopy invasion of erythrocytes by P. knowlesi and observed that the invasion consisted of attachment of the apical end of the parasite to the erythrocyte, deformation of the erythrocyte and entry of the parasite by invagination of the erythrocyte membrane. The entire invasion sequence was complete in approximately 30 seconds.

Ultrastructural studies on invasion were hindered by sampling problems. Recently, Dennis et al. (6) reported a new method for the collection of large

quantities of free, viable merozoites of P. knowlesi which could invade erythrocytes. By applying this method, Bannister et al. (4) confirmed the previous electron microscopic observations on invasion. We have used an approach similar to that of Bannister et al. (4) and report several new findings on the invasion process. They include 1) a junction between erythrocytes and merozoites, 2) movement of the junction during invasion, and 3) the fate of the surface coat on merozoites. These findings will be presented and discussed in this paper.

MATERIALS AND METHODS

Parasitized erythrocytes were obtained from rhesus monkeys (Macaca mulatta) infected with the Malaysian strain of Plasmodium knowlesi (5) when the parasitemia was 10 to 30%. The majority of parasites were schizonts with 8 to 10 nuclei. The blood was mixed with heparin and adenosine diphosphate and passed over glass beads to remove platelets and some white cells. The cells were washed one time at room temperature in culture medium that consisted of medium 199 plus 10 mM glucose, 6.6 mM glycylglycine, 25 ug/ml of Gentamycin, and 10% (v/v) heat-inactivated fetal calf serum. Packed cells were pipetted into 2 mm x 10 cm glass tubes that were sealed at one end. In order to separate the low density schizonts from the other erythrocytes, the cells were spun at 1200 g for 5 minutes. The schizonts formed a brown layer at the top of the tube. The tube was broken at the interface between schizonts and other erythrocytes, and the schizonts were added to the culture medium. Approximately 3×10^9 schizonts were

added to 35 ml of culture medium in the culture chamber and incubated at 37°C.

The culture chamber was built according to the specifications of Dennis et al. (6). The bottom of the culture chamber was covered with a Nucleopore filter with 1.5 um holes. Fresh medium was continuously added through the wall in the side of the chamber and carried out through the filter in the bottom at a rate of approximately 1.5 ml per minute. Merozoites freely passed through the holes in the filter; few schizonts could pass this filter. This permitted collection of viable merozoites soon after their release from schizont infected erythrocytes. One ml of the merozoite suspension (approximately 20,000 merozoites/mm^3) was mixed with 0.1 ml of washed rhesus erythrocytes (100,000/mm^3) and incubated at 37°C with continuous agitation. After one and five minutes, the cell suspension was added to glutaraldehyde fixative (2% glutaraldehyde, 0.05 M phosphate buffer, pH 7.4, and 0.116 M sucrose). Transmission electron microscopy was performed as previously described (1). Thin sections were stained with uranyl acetate and lead nitrate and were examined with a Siemens Elmiskop 101 electron microscope.

In order to visualize the merozoite surface coat clearly, photographic enhancement of the surface coat was accomplished by masking the merozoite image. With the merozoite masked, only the surface coat was projected on the photographic paper. The mask was then removed and the whole image (merozoite and surface coat) was reprojected on the same sheet of paper. The net result was a 1x exposure for the merozoite and a 2x exposure for

the surface coat.

OBSERVATIONS

Since the fine structure of merozoites of P. knowlesi has been reported by several investigators (1, 3, 4, 16), only a brief description of the parasite is presented here (Fig. 1). The entire surface of the merozoite is covered with an electron-dense surface coat measuring ~ 20nm that consists of fine fibrils. Two electron-dense rhoptries and a few electron-dense micronemes are located in the apical region. A mitochondrion and nucleus are located in the posterior portion.

The invasion of the erythrocytes by the merozoites of P. knowlesi is inititated by the merozoite contacting the erythrocyte with the apical end oriented against the erythrocyte membrane (Fig. 1). The erythrocyte membrane at the point of the interaction is slightly raised initially (Fig. 1), but eventually a depression is created in the erythrocyte membrane (Fig. 2). The erythrocyte membrane to which the merozoite is attached becomes thickened (Fig. 2, inset).

As invasion progresses, the depression in the erythrocyte deepens and conforms to the curvature of the merozoite (Figs. 3, 4, 6, 7). At this time the thickened, electron dense zone on the erythrocyte membrane is no longer observed at the point of the initial attachment but now appears at the orifice of the merozoite induced invagination of the erythrocyte membrane (Figs, 3, 4, 5, 6). This thickened area of the erythrocyte membrane measures ~ 15 nm in thickness and ~ 250 nm in length and appears to be a thickening of the inner leaflet of the bilayer (Fig. 5). (The membrane of a normal erythrocyte is 7.5 to 10 nm in thickness.)

The thickened erythrocyte membrane forms a junction with the merozoite. The merozoite membrane often appears pulled toward the thickening in this area (Fig. 5). It is likely that there is a relatively greater shrinkage of merozoite than erythrocyte during specimen preparations. Because of this, the erythrocyte and merozoite remain in contact only at the junction and apical region. The gap between these two membranes is $\sim$ 10 nm and fine fibrils extend between these two parallel membranes. The junction forms a circumferential interaction at the orifice of the invaginated erythrocyte membrane, since it is always located at each side of the orifice by transmission electron microscopy regardless of the plane through which the section passes. When entry is completed (i.e., the merozoite is within the erythrocyte), the junction appears to fuse at the posterior end of the merozoite (Fig. 8), closing the orifice in the fashion of an iris diaphragm. The merozoite membrane still remains in close apposition to the thickened erythrocyte membrane (Fig. 8, inset) at the point of the final closure.

During the invasion process the distribution of the surface coat alters. Prior to invasion, merozoites are covered with an uniform surface coat of $\sim$ 20 nm thickness (Fig. 9). During invasion, no surface coat is visible on the portion of the merozoite within the erythrocyte invagination, whereas the surface coat on that portion of the merozoite remaining outside the erythrocyte appears to be similar to that seen on free merozoites (Fig. 10). No accumulation of the surface coat is seen at or beyond the junction. This becomes more apparent when the surface coat is photographically intensified by a double exposure technique.

Throughout invasion, the apical end remains in contact with the erythro-

cyte. In some specimens, several small vacuoles are seen in the erythrocyte cytoplasm in this region as originally described by Bannister et al. (4). There is an electron-opaque band between the tip of the apical end and the erythrocyte (Figs. 4,11). This band appears to be continuous with the common duct of the rhoptries. The common duct is formed by the meeting of ductules which lead from each of the rhoptries (Fig. 11). The common duct is less electron dense than the rhoptry itself.

DISCUSSION

Host-parasite interaction is of major importance to parasitic protozoa since their survival depends on host cells which supply environmental and nutritional requirements. They cannot live apart from their host cells or host cell nutrients. In recent years, there has been a great interest in the mechanism by which protozoa attach to and enter their host cells. Malaria parasites and other related protozoa such as Toxoplasma (2,13), Babesia (18), Eimeria (12), and Lankesteria (20) enter within an invagination of the host cell membrane, apical end first. In the case of malaria; invasion is probably explained other than by the merozoite pushing its way into a cell or by the cell ingesting the merozoite. The merozoite has limited motility and the host cell, the mature erythrocyte, is non-phagocytic. The observations in the present study provides a possible mechanism for erythrocyte membrane invagination by the merozoite.

Invasion of erythrocytes by merozoites requires a number of distinct steps. They include 1) initial attachment of the merozoites to the erythrocyte membrane, 2) invagination of the erythrocyte membrane around the mero-

zoite to form a parasitophorous vacuole, and 3) sealing of the erythrocyte after completion of merozoite invasion. The initial attachment involves an interaction between the erythrocyte membrane and the apical end of the merozoite. Miller et al. (17) reported that initial recognition and attachment between P. knowlesi merozoites and erythrocytes probably involve specific determinants, probably associated with Duffy blood group related antigens. After contact, a junction forms between the merozoite and host cell. The junction appears to be a circumferential attachment at the orifice of the parasitophorous vacuole as shown in Fig. 12. As the junction moves over the merozoite, the merozoite is brought within the invaginated erythrocyte membrane. The junction fuses at the posterior end of the merozoite and the merozoite is then inside the erythrocyte within a vacuole originating from the inverted erythrocyte membrane.

Are these events occurring during invasion related to endocytotic processes by which phagocytic cells ingest particles, other cells, and microorganisms? Griffin et al. (8) proposed alternative hypotheses for endocytosis of particles, namely, specific attachment triggering endocytosis and zippering. Triggering requires specific receptors for attachment but the ingestion is independent of receptors outside of the attachment zone. Zippering is attachment to receptors around the circumference of the particles. Zippering may require a metabolically active cell, such as macrophage phagocytosis of cells via Fc or C3b receptors (8,9), or may be a passive process, such as envelopment of Sendai virus by ganglioside-containing liposomes (10). Neither model,

however, appears to explain the observation during invasion by malarial parasites.

Therefore, we would propose the following alternative models for invasion of erythrocytes by malarial merozoites, although we have no idea of whether the parasite, the erythrocyte, or both supply the energy for the event. 1) Movement of the junction at the level of the membrane. This movement may be related to the lateral displacement of the junction by the agency of membrane flow, which is now a well established phenomenon (21). However, it should be pointed out that this would necessitate simultaneous parallel flow of membrane components in both cells. 2) Attachment-detachment (modified zipper) model. The junction itself may be capable of migrating on the surface of a relatively stable plasma membrane. This would require that the leading edge of the moving junction becomes attached while the following edge becomes detached, perhaps by enzymatic cleavage.

During invasion alterations occur in the merozoite and erythrocyte membranes within the vacuole. The merozoite coat that is evenly distributed over the merozoite surface before invasion (16) is now missing (4, 15, present study). Although Bannister et al. (4) suggested that the surface coat accumulates at the orifice of the entry site of the erythrocyte by the merozoite, there is no evidence to suggest such an occurrence in our present study. The surface coat beyond the junction appears to be unaltered in density. Therefore, simple capping does not seem to fit our observations.

In the early 1960's, investigators reported by electron microscopy that

the motile forms of these intracellular protozoa possess anterior organelles, rhoptries and micronemes, which were thought to be associated with host cell entry (1). In Plasmodium, Babesia (18), Eimeria (11), and Besnoitia (19), a ductule runs from the rhoptries to the apical end which is the point of initial contact between the protozoa and host cell. During invasion, the lower electron density in the duct suggests a release of rhoptry contents. Kilejian (14) suggested that the rhoptries and micronemes of an avian malarial parasite, P. lophurae contain a histidine-rich protein which invaginates the erythrocyte membrane. Our observation on the connection between the rhoptries and the erythrocyte membrane presented here supports the supposition that the rhoptries play a role in merozoite entry into the erythrocyte. However, the identification of the function of rhoptries and micronemes must await the isolation of the contents of these organelles for the analysis of their chemical and physical properties.

ACKNOWLEDGEMENTS:

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Figure Legends

Fig 1. Electron micrograph of a merozoite of P. knowlesi at the initial contact between the merozoite's apical end (arrow) and an erythrocyte (E). The erythrocyte membrane is slightly raised at the point of the interaction. The merozoite shows an apical end (A), a rhoptry (R), a nucleus (N), and a mitochondrion (M). The surface is covered with a surface coat (double arrow). 50,000X

Fig 2. Electron micrograph of a merozoite (Mz) contacting an erythrocyte (E). The membrane is thickened (15 nm) at the attachment site (arrow). 54,000X. Inset: Higher magnification micrograph of the erythrocyte-merozoite attachment site showing the thickened erythrocyte membrane. 112,000X

Fig 3. An advanced stage of erythrocyte (E) entry by a merozoite (Mz). The invagination of the erythrocyte is created by the merozoite. Note a junctional attachment (C) at each side of the entry orifice. 54,000X

Fig 4. A further advanced stage of erythrocyte (E) entry by a merozoite (Mz). The junction (C) is formed between the thickened membrane of the erythrocyte and merozoite plasmalemma. The invagination deepens and conforms to the curvature of the merozoite. An electron opaque projection connects the apical end and the erythrocyte membrane (arrow). 50,000X

Fig 5. Higher magnification micrograph of the junction showing the thickened erythrocyte membrane and fine fibrils between the two parallel membranes. 144,000X

Fig 6. A further advanced stage of erythrocyte (E) entry by a merozoite (Mz). The junction (C) is always located at the orifice of erythrocyte entry and is now located at the more posterior portion of the merozoite than in Figs 3 or 4. 48,000X

Fig 7. Erythrocyte (E) entry by a merozoite (Mz) is almost completed and a small orifice (arrow) is seen at the posterior end of the merozoite. The junction (C) is now moved to the posterior end of the merozoite. 54,000X

Fig 8. A merozoite (Mz) is now inside of an erythrocyte. However, the posterior end of the merozoite is still attached (arrow) to the thickened erythrocyte membrane, while the other portion is separated from the surrounding membrane by a clear space. 50,000X. Inset: Higher magnification micrograph of the thickened erythrocyte membrane which is attached to the merozoite. 300,000X

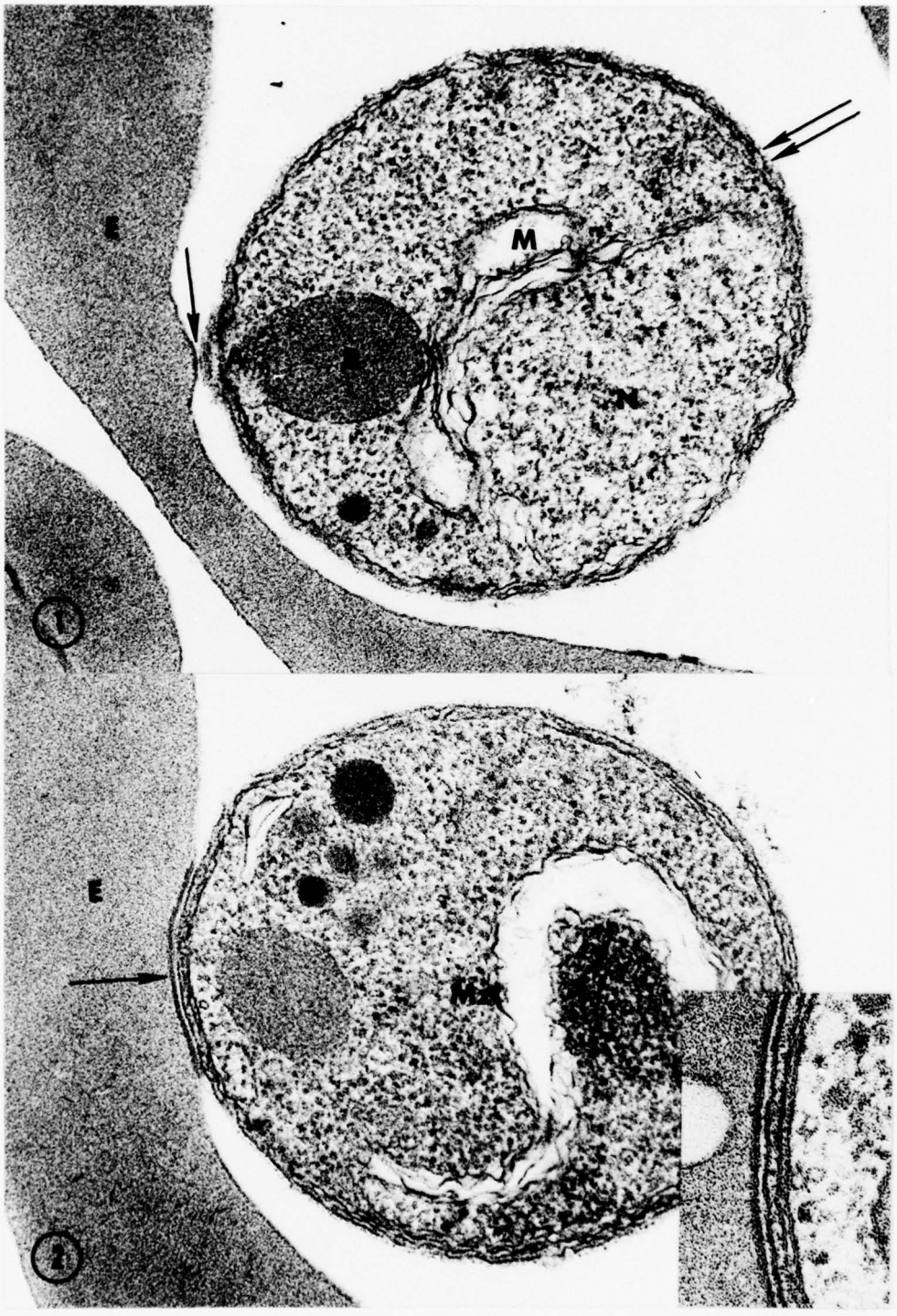
Fig 9. A free merozoite showing a surface coat (arrow) covering the entire surface. 68,000X (This surface coat was photographically intensified by a double exposure technique.)

Fig 10. A merozoite entering an erythrocyte. No surface coat is visible on the portion of the merozoite surface which has invaginated the

erythrocyte membrane, whereas the prominent surface coat is visible behind the attachment site (arrow). No accumulation of the surface coat is seen at the site of the junction. 68,000X. (This surface coat was photographically intensified by a double exposure technique.)

Fig 11. Higher magnification electron micrograph showing two rhoptries (R) at the apical end. The common duct (Cd) is formed by the meeting of ductules which lead from each rhoptry. An electron opaque projection connects the apical end and the erythrocyte membrane (arrow). 120,000X

Fig 12. A diagram of a moving circumferential junction between merozoite and erythrocyte. The moving junction brings the merozoite within an invagination of the erythrocyte membrane.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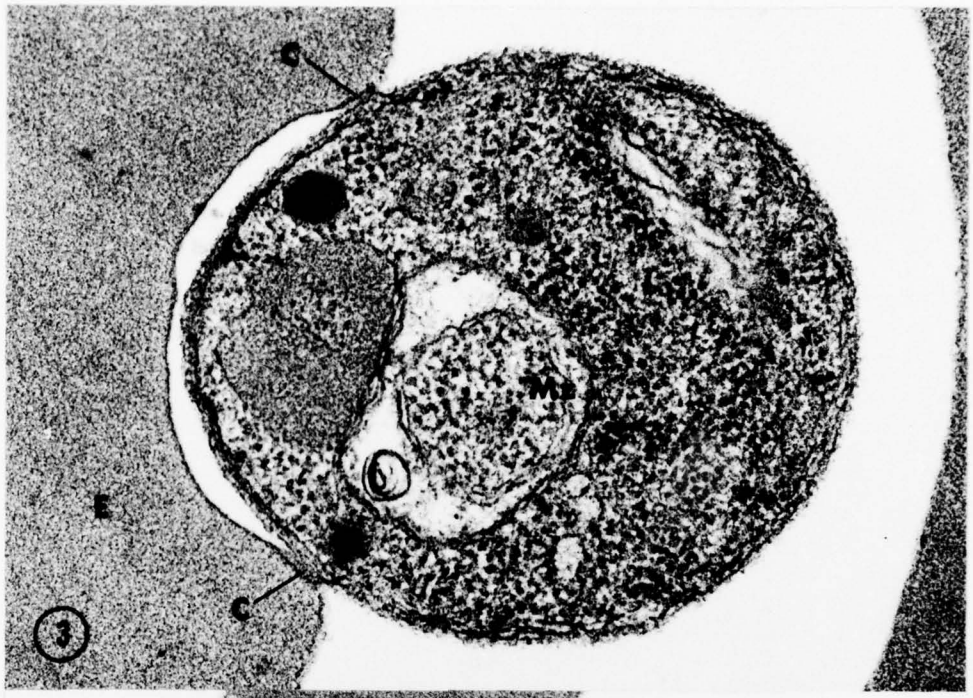

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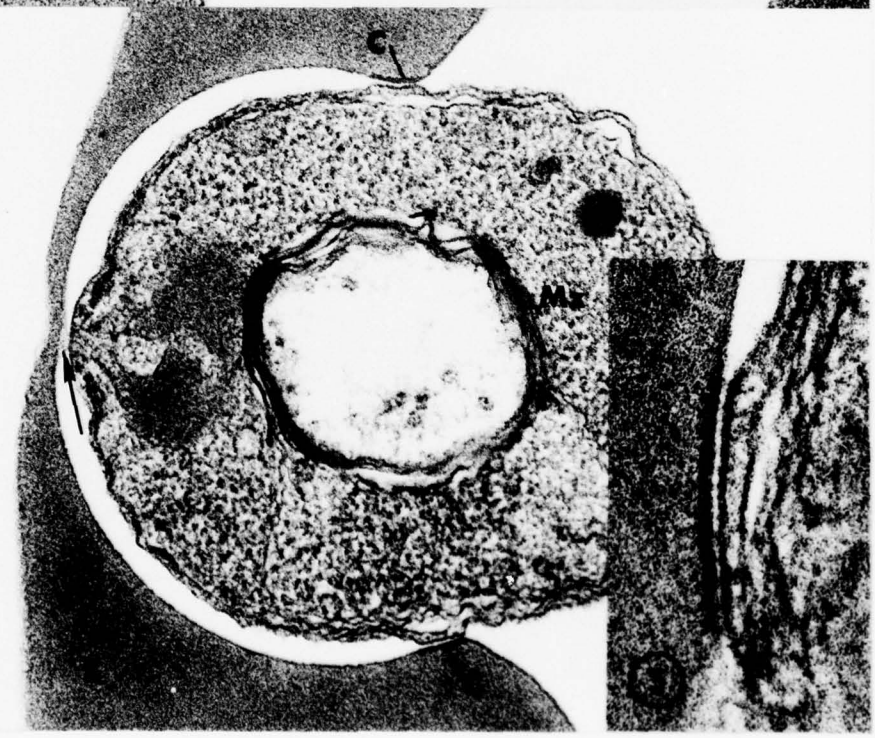

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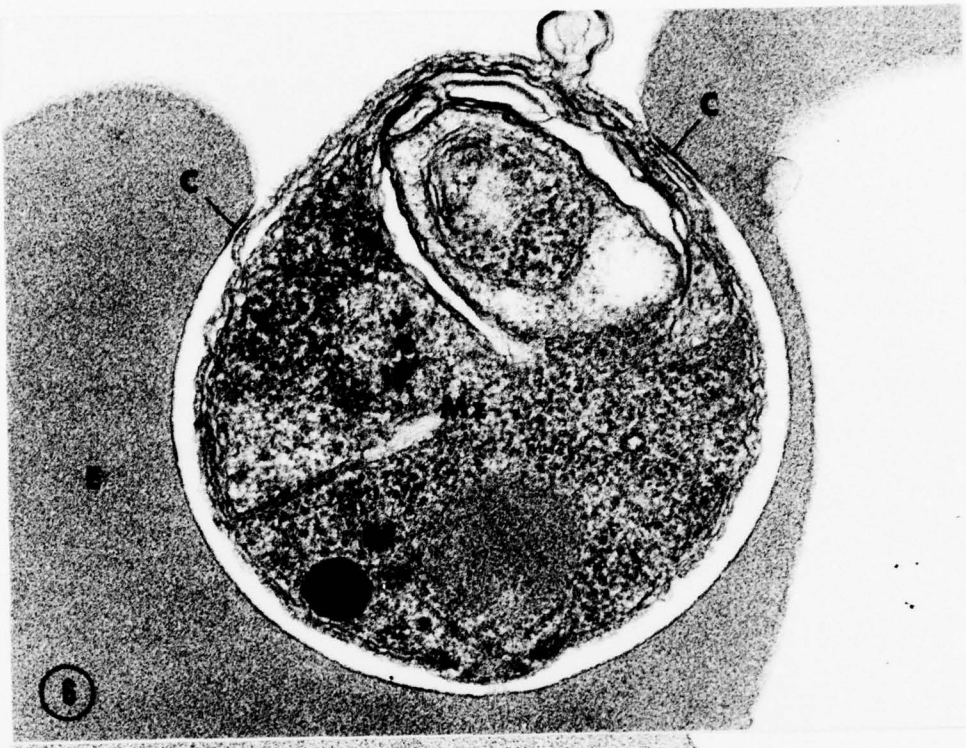
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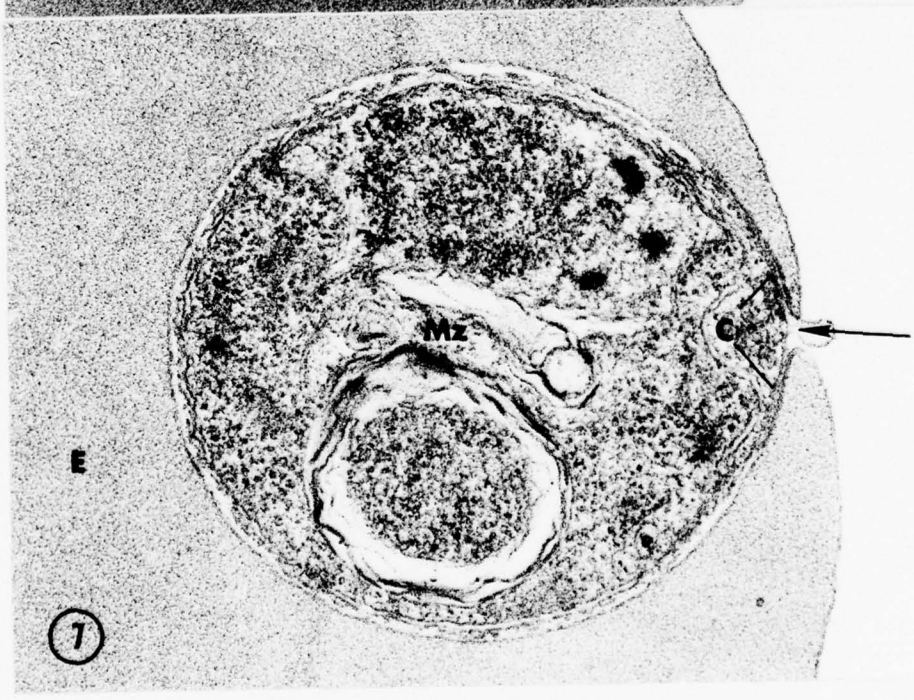
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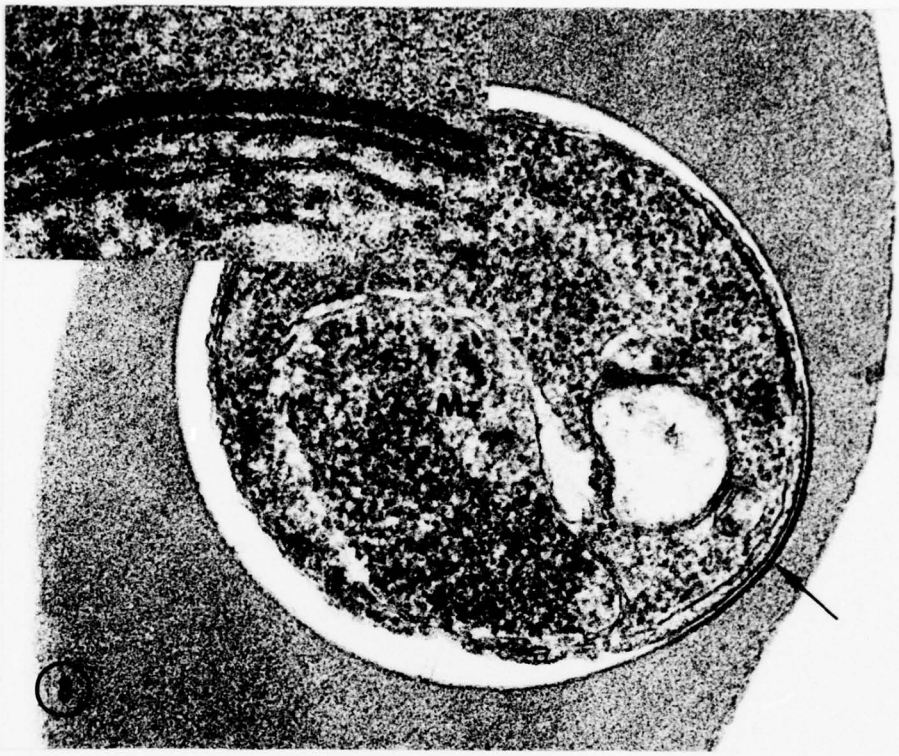

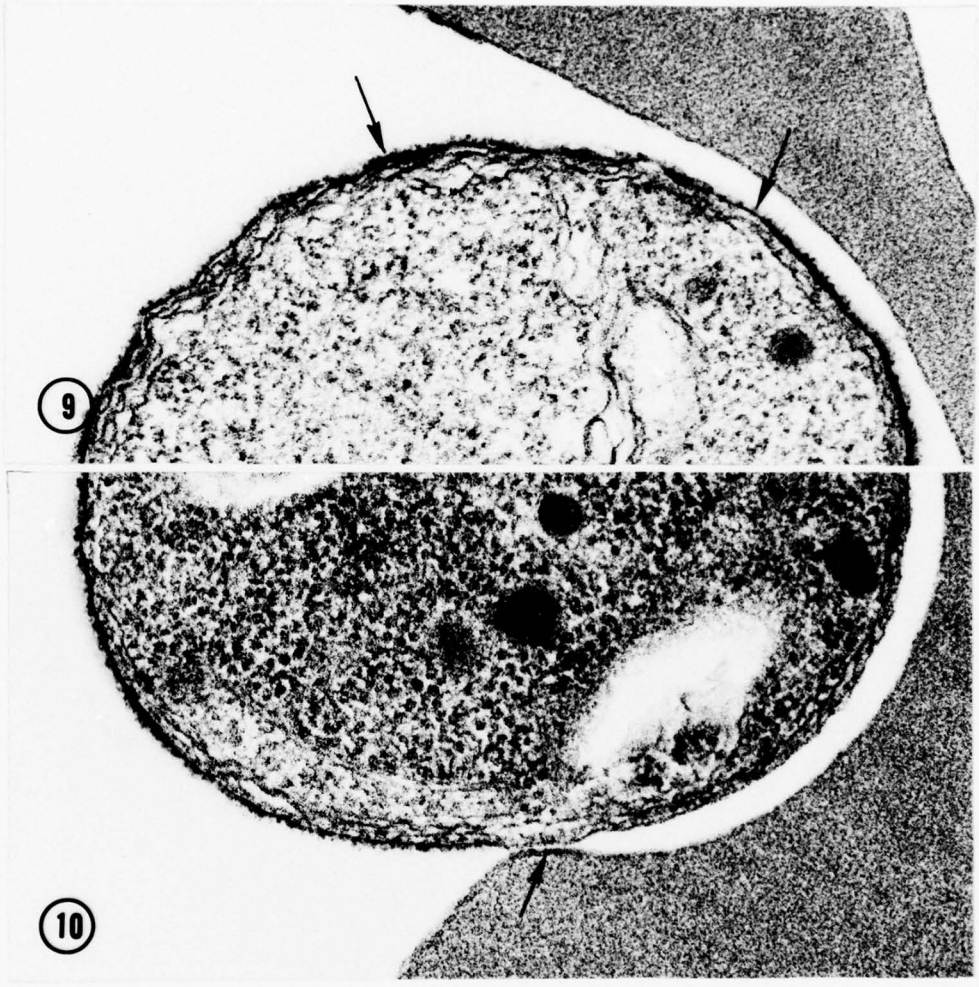
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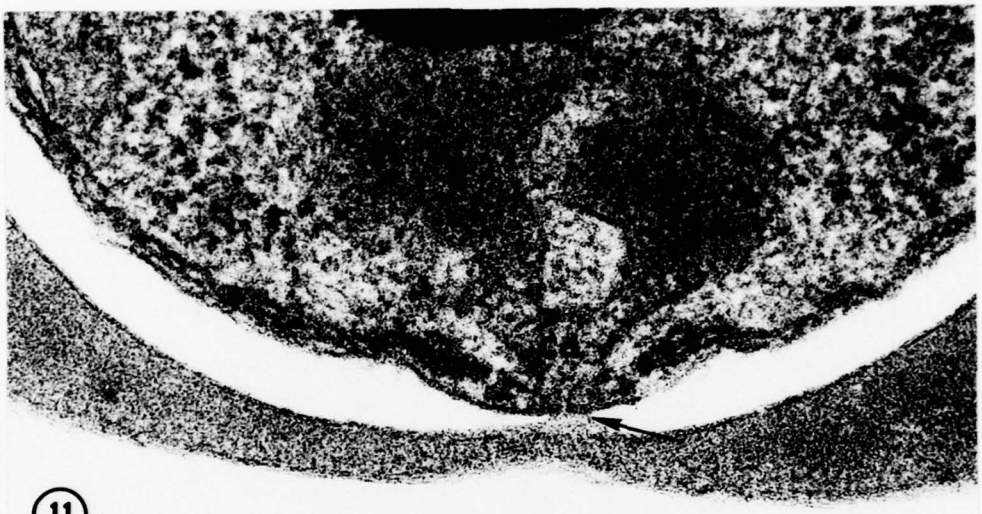

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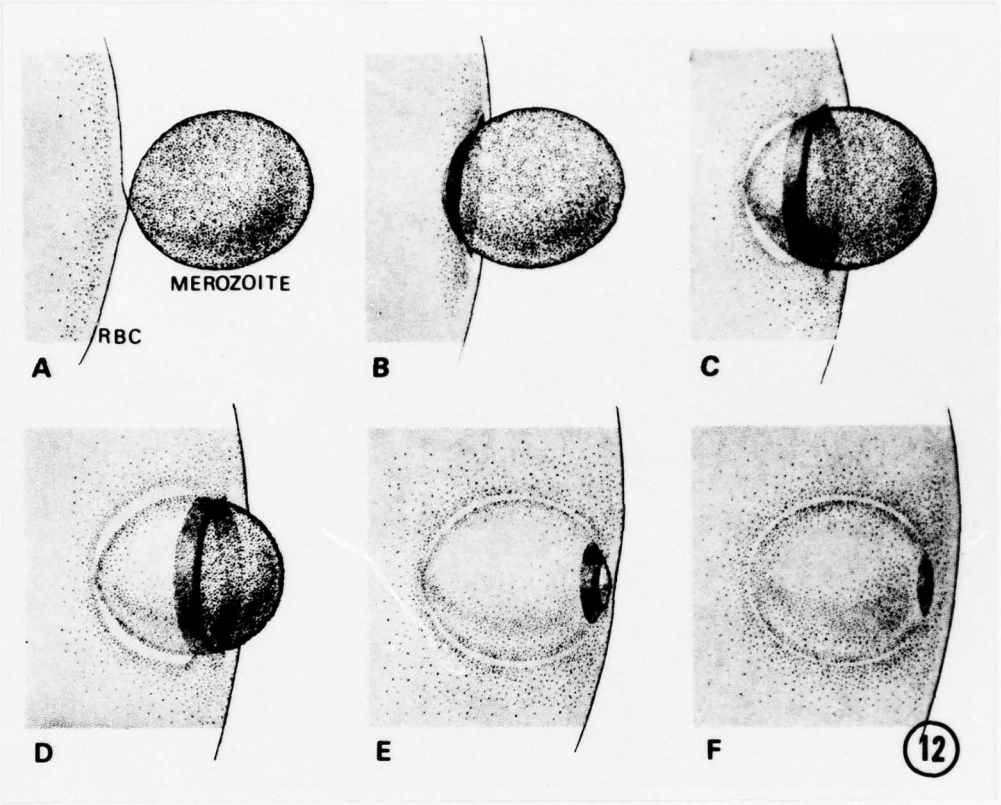

Aikawa et al.

Interaction Between Cytochalasin B-Treated Malarial Parasites and Red Cells: Attachment and Junction Formation

Running Title: Cytochalasin-Treated Merozoites and Red Cells

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Introduction

Malaria parasites develop within red blood cells (RBCs), and at full maturation infected RBCs rupture, releasing individual merozoites that invade other RBCs. Invasion, the process by which the extracellular merozoite becomes established as an intracellular parasite occurs through a sequence of events that include recognition and attachment (1), junction formation (2), invagination of the RBC membrane around the merozoite (2), and finally sealing of the invaginated membrane to form a vacuole and to reestablish continuity of the RBC membrane (2). The meorozoite has specialized organelles, rhoptries and micronemes at the apical end which is in apposition to the RBC during invasion (1-4). A junction forms between the apical region and the RBC membrane (2). As the junction moves over the merozoite, the merozoite is brought within the invaginated RBC membrane. Failure of viable merozoites to invade RBCs may be due to defects at any stage in the invasion process. With the exception of the Duffy negative human RBCs, P. knowlesi merozoites do not attach to or deform RBCs that are refractory to invasion (subprimate and chymotrypsin-treated human RBCs). In case of Duffy negative human RBCs, the only human RBC refractory to invasion by this parasite (5,6), the merozoite on contact induces deformation of the Duffy negative RBC, but instead of entering within an invagination of the RBC, the merozoite detaches and then interacts with other RBCs. Identification of the defect in invasion of Duffy negative RBCs could not be further explored by previously available techniques because of the rapidity of events.

In order to show the structural and functional differences between the normal invasion sequence and the defective interaction between Duffy negative human RBCs and P. knowlesi merozoites, we developed a method for the isolation of the attachment phase of invasion. This method uses cytochalasin-treated

P. knowlesi merozoites. Cytochalasin-treated merozoites attach only to RBCs from susceptible hosts (rhesus monkeys and man) and form a junction between the apical end of the merozoite and the RBC. These treated merozoites will also attach specifically to Duffy blood group negative human RBCs, although these human RBCs are refractory to invasion (5). However, the mechanism of attachment differs in that there is no junction formation with Duffy negative RBCs, suggesting that the defect in invasion of Duffy negative RBCs is at the step of junction formation.

Materials and Methods

Preparation of Plasmodium knowlesi merozoites. Rhesus monkeys (Macaca mulatta) were infected with a Malaysian strain of P. knowlesi. When the parasitemia was between 10% and 35% and the majority of schizonts within RBCs contained 10 or more nuclei, 10 to 20 ml of blood was drawn into a heparinized syringe. All subsequent steps in preparation were carried out at room temperature (23 to 25°C). After one mg/ml of adenosine diphosphate was slowly added to the blood, the blood was mixed for two min and squeezed over glass beads (0.11 mm diameter) that were packed in a 10 ml plastic syringe to remove platelets and some white cells. The blood was diluted in 40 ml of modifed Medium 199 (See 6 for details) and centrifuged for 4 min at 850g. The supernatant was discarded and the packed RBCs were loaded into 2 x 100 mm glass tubes. The tubes were centrifuged for 5 min at 1300g. The low density schizont-infected RBCs formed a brown layer above the uninfected RBCs. The tube was scored and broken at the interface and the brown layer pooled. The yield was approximately 4×10^9 schizont-infected RBCs. The schizont-infected RBCs were added to a culture chamber designed for merozoite collection (7,8). The parasites were allowed to develop in modified Medium 199 at 37°C. As merozoites were released from schizont-infected RBCs, they were collected for attachment and invasion assays.

Merozoite attachment assay. One ml of culture chamber effluent containing 2 to 5 x 10^7 merozoites/ml was collected in a four ml glass vial at room temperature. 0.1 ml of a solution containing cytochalasin B in dimethyl sulfoxide (DMSO) and culture medium was added so that the final concentration of cytochalasin B was 10 µg/ml in 0.1% DMSO. After 3 min incubation, 0.1 ml of RBCs in culture medium (10^8 RBCs/ml) was added, warmed to 37°C for 2 to 4 min with mixing and then centrifuged for 2 min at 1000g. The supernatant was removed and the pellet was resuspended in the final drop of medium. The suspension was allowed to stand for 2 min and then fixed by the addition of 2 ml of buffered glutaraldehyde (2% glutaraldehyde, 0.05 M Na phosphate, pH 7.4 and 0.116 M sucrose). The cells were mixed with pasteur pipette and then allowed to settle overnight.

The number of RBCs with attached merozoites was then enumerated as follows. The cells were suspended in 1 ml of buffered glutaraldehyde with a pasteur pipette. One drop of the cell suspension was placed on a slide and was covered by a 22 x 22 mm cover glass which was elevated at one end by another cover glass. The cells were viewed immediately with Smith differential interference optics (Leitz). Whenever a merozoite was seen in apposition to a RBC, the cover glass was tapped with an applicator stick so that the RBC and merozoite would move in the media. If they moved together, the merozoite was considered attached. In some studies we also determined by interference microscopy if the rhoptries (paired organelles of the merozoite) were in apposition to the RBC or oriented away from the RBC.

The studies on the effects of temperature on attachment and some assays for RBC susceptibility were performed without centrifugation of

merozoites with RBCs. For the temperature experiments, merozoites and RBCs preequilibrated at 37°C, 23 to 25°C and 4°C were mixed continuously at the respective temperature for 4 min before adding the 1 ml suspension to 9 ml of buffered glutaraldehyde. For assay of RBC susceptibility without centrifugation, the merozoite-RBC suspension was mixed at 37°C for 4 min and then fixed in glutaraldehyde.

Invasion assay of cytochalasin-treated merozoites. One ml of culture chamber effluent containing 2 to 5 x 10^7 merozoites/ml was collected in a 4 ml glass vial at room temperature. To the vial was added cytochalasin B in DMSO so that the final concentration was 10 µg, 1 µg, or 0.1 µg in 0.1% DMSO: the mixture was incubated at room temperature for 3 min. As a control, 0.1% DMSO or nothing was added to vials of merozoites. After room temperature incubation, the vial was warmed briefly to 37°C and 0.1 ml uninfected rhesus RBCs in modified Medium 199 (10^8 RBCs/ml was added. The vial was capped and rocked end-to-end (Aliquot Mixer, Ames Company) at 37°C for 30 min, and then placed on ice. The suspension was centrifuged 15 sec. at 1000g, and a thin film was made from the pellet. The percent of RBCs with ring forms was determined on Giemsa-stained smears.

Attachment and invasion of enzyme-treated RBCs. Chymotrypsin (1 mg/ml) treated Duffy blood group positive and negative human RBCs were tested for susceptibility to invasion in Linbro wells (as described previously (6)) and to attachment. The attachment between trypsin (1 mg/ml) treated Duffy negative RBCs and cytochalasin-treated merozoites was studied by electron microscopy. Trypsin (Worthington, TR(3BA)), soybean trypsin inhibitor (Worthington, SI 56H570), chymotrypsin (Worthington, CDS 55J 402X) and phenylmethylsulfonyl fluoride (Sigma, Lot 64C-0335) were incubated with the

RBCs as described previously (6).

Electron Microscopy. RBCs and attached cytochalasin B-treated merozoites were added to glutaraldehyde fixative (2% glutaraldehyde, 0.05 M phosphate pH 7.4 and 0.116 M sucrose). The samples were stained with uranyl acetate and lead nitrate and were examined with Siemens Elmiskop 101 electron microscope.

To visualize the filamentous attachment between merozoites and Duffy negative RBCs photographic enhancement of the filaments was accomplished by masking the merozoite and RBC and by projecting only the filament on the photographic paper. The mask was removed and the whole image was reprojected onto the same sheet of paper. The net result was a 1x exposure for the merozoite and RBC and a 2x exposure for the filament.

In a few experiments, the merozoite-RBC preparation after fixation in glutaraldehyde and postfixation in 1% osmium tetroxide was suspended in 0.4 ml 10% albumin in an attempt to stablilize the attachment to Duffy negative RBCs. 50 µl of 8% glutaraldehyde was added and the sample was centrifuged before the albumin polymerized. The pellet was dehydrated in alcohol and prepared for thin section electron microscopy as described previously (2).

Results

In order to study the complicated series of events leading to invasion, we developed a method for selectively blocking invasion at the attachment phase. Cytochalasin B-treated P. knowlesi merozoites attach to but do not invade rhesus RBCs (Table I, Fig. 1). Since it is known that cytochalasin B affects microfilaments (9), glucose transport (10), and possibly other functions and has a low and high affinity binding site on the RBC (11), we did not attempt to define the mechanism of action of cytochalasin in this system. Instead, we used it operationally to separate the early steps in the interaction between merozoites and RBCs. Because 10 µg/ml of cytochalasin B gave the most marked reduction in invasion, this concentration was used for all

subsequent experiments.

Temperature dependence of attachment. Cytochalasin B-treated merozoites were mixed with rhesus RBCs at 4°C, 25°C and 37°C for 4 min. The cells were fixed with glutaraldehyde and the number of attached merozoites were counted. The highest rate of attachment was at 37°C; few attached at 4°C (Table II). In order to increase the attachment rate, the mixture of merozoites and RBCs were centrifuged at 1000g for 2 min after a 4 min incubation at 37°C (See methods for details). Centrifuged samples had as much as 10 fold increase in attachment to rhesus and human RBCs as the uncentrifuged samples.

Specificity of attachment. The assay for attachment was specific in that merozoites (with or without centrifugation), did not attach to guinea pig or avian RBCs. These animals are resistant to infection by P. knowlesi and the merozoites do not interact with or invade the RBCs on direct observation by interference microscopy. In addition to specificity, the assay detects differences in attachment affinity. Attachment to rhesus RBCs was consistently greater than to human RBCs (Table III).

Attachment to Duffy blood group positive and negative human RBCs.

P. knowlesi merozoites invade all human RBCs except Duffy blood group negative RBCs (5,6). Without cytochalasin B in the merozoite-Duffy positive RBC suspension, merozoites in glutaraldehyde-fixed preparations were in all stages of invasion. Without cytochalasin in the merozoite-Duffy negative RBC suspension, few merozoites in glutaraldehyde-fixed preparation of the suspension (with or without 0.1% DMSO) were attached to RBCs and none was invading. With cytochalasin, it was observed for the first time that the attachment rate of P. knowlesi merozoites to Duffy positive and negative RBCs was the same (Table III), despite the resistance of Duffy negative RBCs to invasion. That the Duffy associated event in the invasion sequence was not related to the attachment was further tested by treatment of

Duffy positive RBCs (Fy^a), with anti-Fy^a, a treatment that markedly reduces invasion (5). The attachment rate to antibody-coated RBCs was the same as to untreated RBCs.

Chymotrypsin treatment of Duffy positive or negative RBCs eliminates all interaction with merozoites as observed by interference microscopy (12). Cytochalasin-treated merozoites also cannot attach to these chymotrypsin-treated cells. These cells are comparable to RBCs from refractory sub-primates in that there is no interaction or attachment to them.

Observations on attachment by interference microscopy. Rhoptries are specialized organelles at the apical end of the merozoite and appear as a single or double teardrop-shaped structure. During invasion, the apical end containing the rhoptries are oriented toward the RBC before RBC deformation begins (1). In the present study, cytochalasin-treated merozoites remained attached to RBCs and did not invade them. We determined the orientation of these merozoites relative to the RBC, i.e., the position of the rhoptries in merozoites that were attached to the convex edge of RBC (Fig. 1, Table IV). Those merozoites that were in the concave portions of the RBC (where the merozoite orientation to the RBC could not be determined) or those that had no visible rhoptries were excluded from the analysis. In the majority of cases, the end of the merozoite containing rhoptries (apical orientation) was in apposition to the RBC (Fig. 1, Table IV). The percentage of merozoites with apical orientation was similar for rhesus RBCs, human Duffy positive RBCs and human Duffy negative RBCs (Table IV), although, in any experiment, more merozoites attached to rhesus than human RBCs (Table III).

Electron microscopy. When rhesus RBCs and cytochalasin B-treated merozoites were incubated together, the apical end of the merozoite attached

to the rhesus RBC membrane (Figs. 2 and 3) in a manner identical to that of merozoites without cytochalasin B treatment (2). The RBC membrane to which the cytochalasin-treated merozoite was attached became thickened and formed a junction with the plasmalemma of the apical portion of the merozoite and invaginated slightly to cover the apical end. However, the invasion processes did not advance further and no movement of the junction between the RBC and merozoite occurred. In some sections, vacuoles surrounded by a unit membrane appeared in the RBC cytoplasm near the attachment site (Fig. 3). The vacuoles varied in size. Some resembled pinocytotic vesicles that appeared to be in contact with the RBC membrane; others were elongated and large measuring about 0.8 μm in length and 0.1 μm in width. The contents of these vacuoles were electron translucent, although a few contained fine granular material.

Attachment of the treated merozoites to human Duffy positive RBCs was similar to that described for rhesus RBCs. The apical end of the merozoite attached to the RBC formed a junction with the thickened RBC membrane (Fig. 4), but no further steps in the invasion process took place. Also, vacuoles were observed in the RBC cytoplasm near the site of the merozoite attachment.

In contrast to what was observed with rhesus and Duffy positive human RBCs, we observed attachment but no junction formation between Duffy negative RBCs and cytochalasin-treated merozoites. This despite the fact that we studied four thin sections from each sample of eight experiments. The total number of RBCs and merozoites examined was about 12,000 and 6,000, respectively. The apical ends of many merozoites were orientated towards the RBCs, but instead of a junction, the RBC was about 120 to

160 nm away from the RBC, connected by thin filaments measuring 3 to 5 nm (Fig. 5). The filaments originated from the edge of the truncated, cone-shaped apical end, such that two distinct filaments could be seen in thin sections. This suggests that these filaments are arranged in a cylindrical fashion in three dimensions. Such filamentous attachments between cytochalasin-treated merozoites and RBCs were not observed in the experiments with normal rhesus or human Duffy positive RBCs, but they were observed with schizont-infected rhesus RBCs.

Inasmuch as a junction was not observed between the merozoite and the Duffy negative RBC, it seemed possible that merozoites which were in contact with the RBC membrane became separated from this membrane during specimen preparation. In order to attempt to avoid such detachment during specimen preparation, 10% albumin was added to the glutaraldehyde-osmium fixed specimens before dehydration (See methods). Still, merozoites from this preparation did not form a junction with the RBCs. Merozoites were connected to the RBCs by thin filaments which became more prominent after albumin treatment (Fig. 5, inset).

Previously we demonstrated that trypsin treatment of Duffy negative RBCs made them more susceptible to invasion by P. knowlesi merozoites (6). It was of interest, therefore, to see whether a junction forms between these RBCs and cytochalasin-treated merozoites. The results from these experiments were similar to those obtained in studies with rhesus and Duffy positive RBCs. There was junction formation between merozoites and RBC (Fig. 6). The connection of rhoptries with RBC membrane was also observed (Fig. 6, inset).

Discussion

It was observed that cytochalasin B-treated merozoites attach to rhesus RBCs but cannot enter within these RBCs (i.e., they are not interiorized

within an invaginated RBC membrane). Because cytochalasin has multiple effects on cells (9,10), we made no attempt to relate altered invasion to its known effects. Rather, it was used operationally to further study the early phase of invasion, i.e., attachment of merozoites to the RBC membrane.

Attachment of cytochalasin-treated P. knowlesi merozoites to RBCs from susceptible hosts (rhesus monkeys and man) is specific in that these merozoites will not attach to RBCs from nonsusceptible species (guinea pigs and chickens). Furthermore, the assay is not only species specific but also can detect difference in attachment affinity. There was a significantly greater percentage attachment to rhesus RBCs than to human RBCs, corresponding to the higher invasion of rhesus RBCs. In addition to specificity, the process is temperature sensitive with the greatest attachment at 37°C and little attachment at 4°C.

As in normal invasion (1-4), the majority of attached cytochalasin-treated merozoites were oriented such that the apical region (the region with the rhoptries) was in apposition to the RBC, although a few attached in other orientations. The mechanism of apical orientation is unknown and could relate to receptor density, receptor distribution on the merozoite or the contraction of the merozoite towards the apical region. By thin section electron microscopy, we observed junction formation between the apical region of cytochalasin-treated merozoites and the RBC membrane. As in normal invasion (2), the RBC membrane attached to the merozoite appears thickened. Although movement of the junction around the merozoite (the normal invasion sequence (2)) does not occur after cytochalasin treatment, invaginated vacuoles, probably of the RBC membrane origin, are present in

the apical region. We speculate that the substance released by the rhoptries induces the RBC membrane to flow past the point of the junction forming the observed invaginated vacuole around the apical region. The parasite might have moved within the space formed by the invaginated vacuole except that cytochalasin inhibited movement of the junction around the merozoite. One possible mechanism for the formation of invaginated vacuoles in the RBC might be similar to that proposed for bleb formation after the addition of basic proteins to RBC ghosts (13). These blebs contained little protein and had no intramembrane particles by freeze fracture. It was thus proposed that precipitation of spectrin by basic proteins would compress the intramembrane particles and force the phospholipids to form protein-free lipid vesicle blebs. In the case of invagination of the RBC membrane in malaria, Kilejian has isolated a polyhistidine granule from P. lophurae that may be related to the rhoptry secretion (14,15), and McLaren et al have presented preliminary evidence that the vacuolar membrane is devoid of intramembrane particles (16). We speculate that the rhoptry secretion causes movement of the RBC membrane past the junction to form the vacuolar membrane into which the parasite moves. The moving junction on the parasite brings it into the vacuole.

In addition to the study of normal invasion, the isolation of the attachment phase of cytochalasin-treated merozoites provided a tool for exploring the defect in invasion of Duffy negative RBCs. P. knowlesi merozoites invade all human RBCs except Duffy blood group negative RBCs (5,6). Study of this exception to general susceptibility of human RBCs has helped in the understanding of normal invasion. It appears that a Duffy associated antigen is involved in invasion of human RBCs, and removal or blockage of this antigen reduces invasion (5). Untreated merozoites

deform Duffy negative RBCs on contact, but the merozoites are unable to enter within the invaginated RBC membrane. Instead, after initial interaction (widespread deformation of the RBC membrane), the merozoite detaches from the RBC and can interact with other RBCs (5). However, the relative affinity of merozoites for attachment to Duffy positive and negative RBCs and the nature of the attachment was unknown.

Because of the difference in the interaction with Duffy positive and negative RBCs, we were surprised to find that cytochalasin-treated merozoites attached equally well to Duffy positive and negative RBCs (Table III). In addition, the apical orientation for attachment was the same for Duffy positive and negative RBCs (Table IV). It appears that P. knowlesi merozoites do not attach initially to Duffy associated determinants. Support for this came from the observation that anti-Fy^a coated Fy^a (Duffy blood group a positive) RBCs had normal rates of attachment, even though anti-Fy^a markedly reduced invasion of Fy^a RBCs (5).

Although the attachment of cytochalasin B-treated merozoites to Duffy positive and negative RBCs appears the same by light microscopy, ultrastructural studies identified an important difference in the basis for attachment. No junction was observed between cytochalasin-treated merozoites and Duffy negative RBCs. Instead, filaments arranged in a cylindrical fashion extended from the edge of the apical end of merozoites to the RBC. Trypsinization of Duffy negative RBCs, a treatment that makes these cells susceptible to invasion by P. knowlesi, permits junction formation with cytochalasin-treated merozoites. The data on attachment and junction formation with various RBC preparations are summarized in Table V, and the association of these events with merozoite induced

deformation and invasion is shown. Whenever there is deformation of RBCs on contact with merozoites (see (1) for description of the event), cytochalasin-treated merozoites attach to RBCs. Invasion is only observed after junction formation takes place.

From our results, it appears that attachment and apical orientation is independent of the Duffy associated antigen. The nature of this RBC receptor is unknown, although we have shown that it is destroyed by chymotrypsin treatment of the RBC. The qualities of the parasite that determine apical orientation are also unknown. The absence of junction formation with Duffy negative cells may indicate that the Duffy associated antigen acts as a second receptor for junction formation or, alternatively, a determinant on Duffy negative RBCs blocks junction formation. Chemical characterization of the RBC and merozoite determinants involved in the early steps in invasion should facilitate an understanding of these events and suggest methods for blocking them, thus interfering with the asexual malaria cycle and clinical disease.

Summary

We have previously demonstrated that invasion of RBCs by malaria merozoites follows a sequence: recognition and attachment in an apical orientation associated with widespread deformation of the RBC, junction formation, movement of the junction around the merozoite that brings the merozoite into the invaginated RBC membrane, and sealing of the membrane. In the present paper, we describe a method for blocking invasion at an early stage in the sequence. Cytochalasin-treated merozoites attach specifically to host RBCs, most frequently the apical region that contains

specialized organelles (rhoptries) associated with invasion. The parasite then forms a junction between the apical region and the RBC. Cytochalasin blocks movement of this junction, a later step in invasion.

Cytochalasin-treated (P. knowlesi) merozoites attach to Duffy negative human RBCs, although these RBCs are resistant to invasion by the parasite. The attachment with these RBCs, however, differs from susceptible RBCs in that there is no junction formation. Therefore the Duffy associated antigen appears to be involved in junction formation, not initial attachment.

Acknowledgements

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Table I

The effect of cytochalasin (cyto) B treatment of merozoites on invasion of rhesus red cells

Treatment	Percent Invasion		
	Exp. 1	Exp. 2	Exp. 3
None	15.1	2.7	3.8
DMSO (0.1%)	7.5	1.4	1.8
Cyto B (10 µg/ml)	0.8	0	0
(1 µg/ml)	1.5	0	0.3
(0.1 µg/ml)	22.5	4.4	0.9

Table II

The effect of temperature on attachment of cytochalasin B-treated merozoites to rhesus red cells. (Data are presented as per cent of RBCs with merozoites attached.)

	Exp.1	Exp. 2	Exp.3
37°C	8.9	1.3	2.5
23-25°C	1.5	1.4	0.4
4°C	0.2	0.1	0.3

Table III

The effect of red cell type on attachment by cytochalasin B treated merozoites. (Data are presented as per cent of RBCs with merozoites attached.)

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Rhesus RBCs	7.3	33	4.7	17.6	
Human RBCs*					
a. Duffy positive	1.3	8.6	0.8	14.1	1.5
	1.3	5.2	0.8	4.3	
b. Duffy negative	0.5	10.6	1.5	2.9	1.2
	0.7	8.2	1.5		

* Multiple samples run in a particular experiment are from different individuals.

TABLE IV

The orientation of cytochalasin-treated merozoites attached to RBCs

Type of RBC	Centri-* fugation	Orientation of Attachment (%)†			Numb Coun
		Apical	Not Apical	Indeterminate	
Rhesus	No	52.1	4.2	43.7	71
	Yes	70.2	3.5	26.3	57
Human Duffy Positive	No	49.2	13.6	37.3	59
	No	26.3	5.3	68.4	19
	Yes	72.9	4.7	22.4	85
	Yes	50.0	0	50.0	52
Human Duffy Negative	No	44.8	6.0	49.3	67
	No	50.0	1.9	48.1	52
	Yes	46.2	0.9	52.8	106
	Yes	44.4	1.4	54.2	72

* Half of the RBC-merozoite mixture was centrifuged before fixation; the other half was fixed without centrifugation.

† The orientation is determined on merozoites attached to the convex surface of the RBC (apical, rhoptries next to the RBC; not apical, rhoptries in any other orientatio (See Fig. 1). Indeterminate means that the merozoite is attached to the concave surface of the RBC or the rhoptries are not visualized.

‡ The total number of attached merozoites that were counted.

TABLE V

Attachment and invasion of red cells (RBCs) by Plasmodium knowlesi merozoites

	Attachment Cytochalasin B	Deformation*	Junction Formation	Invasion
Rhesus RBCs	+	+	+	+
Duffy Positive (human RBCs)	+	+	+	+
Duffy Negative (human RBCs)	+	+	-	-
Duffy Negative Trypsin Treatment	+	+	+	+
Human RBCs Chymotrypsin Treatment	-	-	-	-
Guinea Pig RBCs	-	-	-	-

+ present; - absent

* Contact between the apical end of viable P. knowlesi merozoites and RBCs from man or monkeys results in a rapid and marked deformation of the RBC (1).

Figure Legends

Figure 1 Interference microscopy photograph showing the attachment between cytochalasin B-treated merozoites and erythrocytes (RBCs). The apical end of the merozoite can be identified by the presence of the rhoptry (arrow). A) Attachment between the apical end of a merozoite and a rhesus RBC. B) Attachment between the side of a merozoite and a rhesus RBC. Note a rhoptry is present in the center of the merozoite. C) Attachment between a merozoite and a Duffy blood group positive human RBC. D) Attachment between a merozoite and a Duffy negative human RBC. X2,500.

Figure 2 Electron micrograph showing the attachment between the apical end of a cytochalasin B-treated merozoite (M) and rhesus erythrocyte (E). The erythrocyte membrane is thickened (double arrow) at the attachment site. A few vacuoles (V) are seen in the erythrocyte cytoplasm and some of them are in contact with the invaginated erythrocyte membrane (arrow). X50,000.

Figure 3 Electron micrograph showing the attachment between a cytochalasin B-treated merozoite (M) and a rhesus erythrocyte (E). Note several vacuoles (V) in the erythrocyte cytoplasm. X57,000.

Figure 4 Electron micrograph showing the attachment between the apical end of a cytochalasin B-treated merozoite (arrow) and a Duffy positive human erythrocyte. Several elongated vacuoles (V) are present near the attachment site. X50,000.

Figure 5 Electron micrograph showing that a cytochalasin B-treated merozoite is connected with a Duffy negative human erythrocyte by two fine fibrils (arrow) which are extending from the edge

of the apical end. The fibrils were photographically intensified by double exposure technique. (See methods.) X50,000.

Inset: Two fine fibrils connecting the apical end of a merozoite and a Duffy negative human erythrocyte. This specimen was treated with 10% albumin before preparation for EM embedding (See methods). X78,000.

Figure 6 Electron micrograph showing the attachment between the apical end of a cytochalasin B-treated merozoite and a trypsin-treated Duffy negative human erythrocyte. The erythrocyte membrane is thickened (double arrow) at the attachment site. An electron opaque projection (arrow) is extending from the rhoptry through an opening in the apical end to the erythrocyte membrane. X51,000.

Inset: Higher magnification showing the projection connecting between the apical end and the erythrocyte. A portion of the rhoptry (R) is less electron dense. X79,000.

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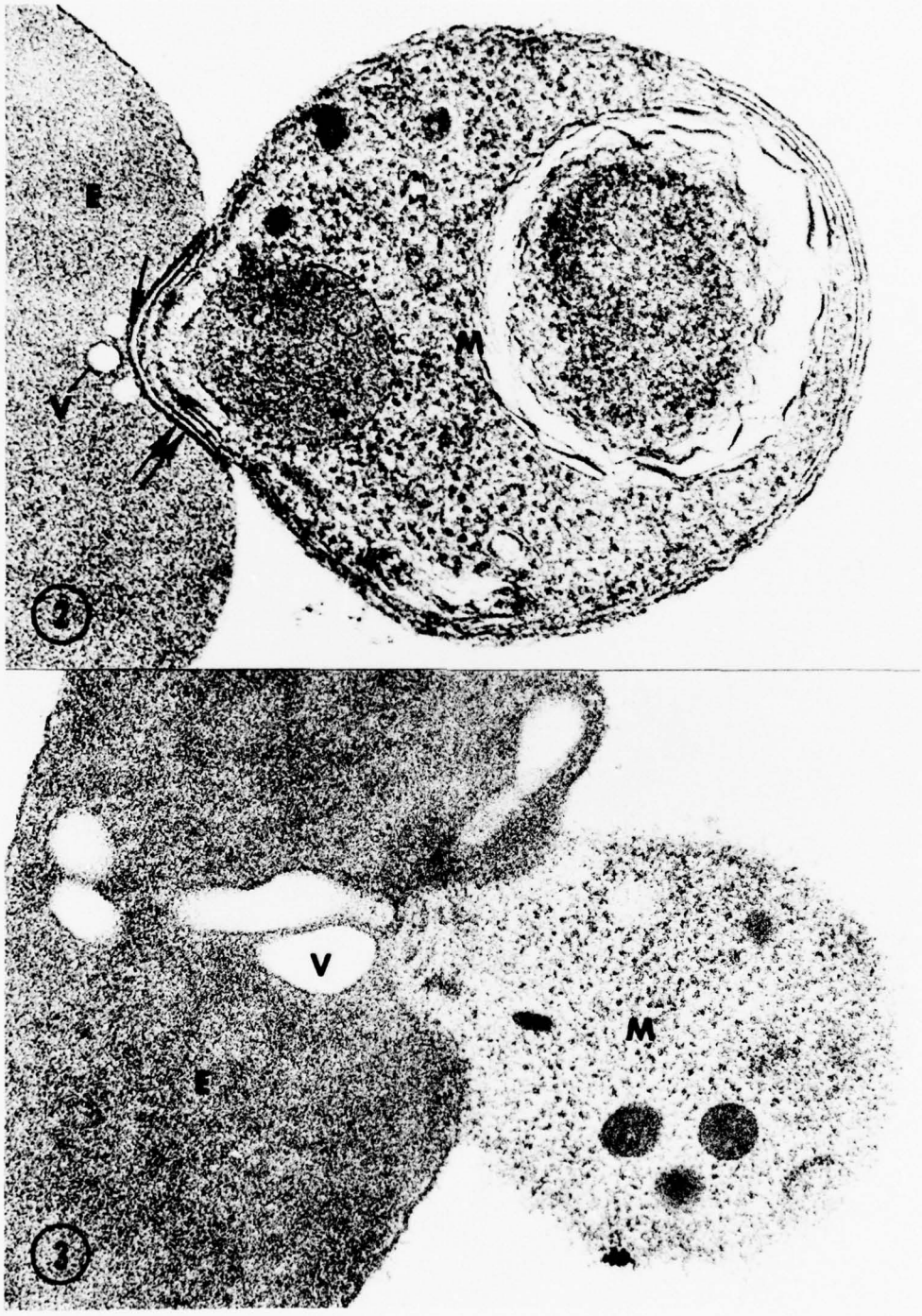
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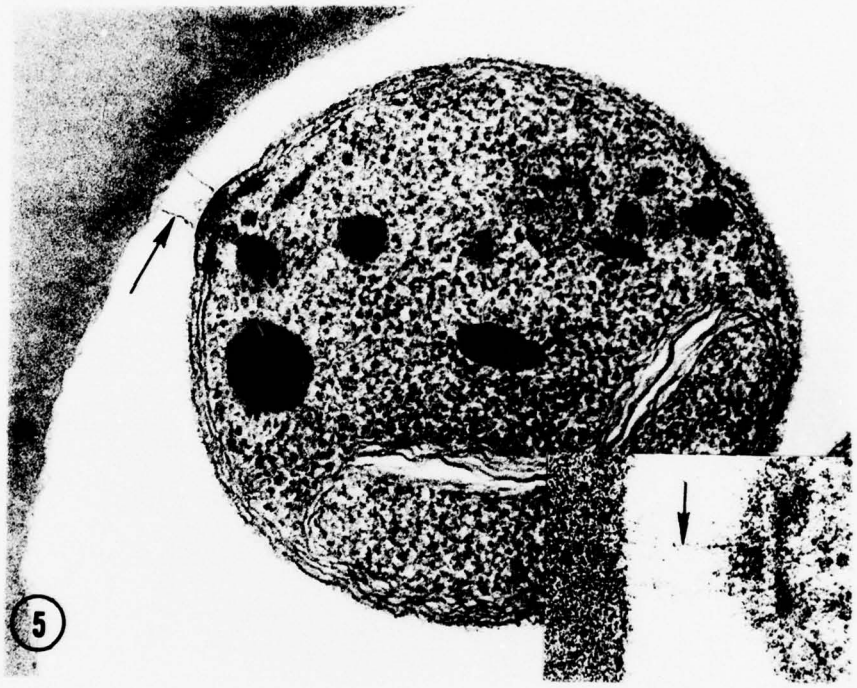
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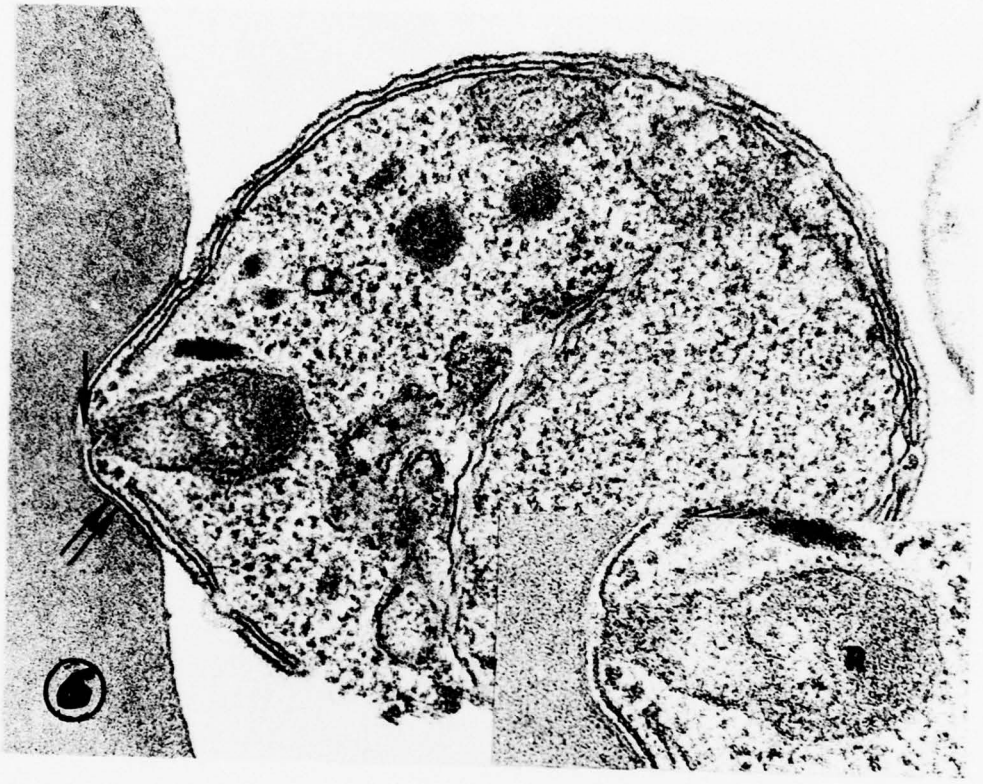

Freeze fracture study on normal and antibody-treated malarial sporozoites

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Synopsis

Freeze fracture of P. cynomolgi, P. knowlesi, and P. berghei sporozoites before and after incubation with immune serum were studied by electron microscopy. There are evenly distributed numerous intramembranous particles (IMP) on the P face of the outer membrane. The E face of the plasma membrane shows fewer IMP than the P face of the plasma membrane. The E face of the intermediate membrane has few IMP and shows linear slightly raised ridges along the long axis of the parasite. The P face of the intermediate membrane shows many IMP which are aligned along the long axis of the sporozoite. On the P face of the inner membrane, IMP are aligned in very distinct rows conforming to the long axis of the parasite. The E face of the inner membrane shows a few randomly distributed IMP.

A prominent change in the sporozoite incubated in immune serum is the appearance of a layer of particle aggregates surrounding the parasite. The P face of the plasma membrane shows several clear areas devoid of IMP as well as IMP aggregates. No changes are detectable in the other fractured faces of the pellicle. This observation suggests that immune serum only acts on the P face of the plasma membrane.

Introduction

Protection against malaria infection occurs in animals immunized with irradiated sporozoites of malarial parasites (9). Although the mechanism of this protection is still not fully understood, it appears to be in part antibody mediated. Ultrastructural studies by thin sectioning on sporozoites of P. cynomolgi and P. berghei incubated with the respect antisera indicated a prominent, thick coat surrounding the outer membrane of the sporozoites, whereas sporozoites incubated with normal sera or media 199 did not show any alterations (2). Therefore, it seems apparent that antigen-antibody reaction occurring at the surface of the sporozoite plays an important role in malaria immunity. However, transmission electron microscopy on thin sections alone cannot detect precise structural alteration of the sporozoite membrane. The freeze fracture technique appears to be the only direct morphologic method for the study of plasma membrane alterations. Recently Douglas (3) demonstrated the redistribution of intramembrane particles (IMP) of pulmonary alveolar macrophages after interaction with IgG and IgM suggesting that there is extensive plasma membrane reorganization after specific interaction between immunoglobulin and the plasma membrane.

Although there are a few reports of freeze fracture studies on protozoa such as Toxoplasma (10), Eimeria (4), and Sarcocystis (10) and the erythrocytic stages of Plasmodium (7,8), no freeze fracture report on malaria sporozoites is available. We undertook to investigate the freeze fracture of P. cynomolgi, P. knowlesi, and P. berghei sporozoites before and after incubation with immune serum and the results are compared to determine the effects of immune serum on IMP distribution of these sporozoites.

Materials and Methods

Animals. Female A/J mice (Jackson Memorial Laboratories, Bar Harbor, Maine) of at least 8 weeks of age were used for immunization with irradiated sporozoites of P. berghei. Juvenile rhesus monkeys (Macaca mulatta) were imported from northern India and conditioned by Hazelton Prime labs, Farmingdale, New Jersey. These animals were immunized by the bite of irradiated P. cynomolgi-infected mosquitoes or inoculated with cynomolgi malaria and used as a source of infective blood meals for mosquitoes.

Parasite strains and their propagation. Laboratory-bred Anopheles stephensi were infected with either the NK 65 strain of P. berghei or the B strain of P. cynomolgi. The former has been maintained in golden hamsters (Cricetus auratus) by weekly blood passage, alternating with frequent cyclical transmission through mosquitoes. P. berghei-infected mosquitoes were held at 21°C under conditions optimal for sporozoite development (12). The P. cynomolgi B strain has been maintained in rhesus monkeys by i.v. inoculation of either sporozoites or blood stages. P. cynomolgi-infected mosquitoes were maintained at 25°C. Anopheles bababacensis infected with P. knowlesi were obtained from Dr. Robert Gwadz, Laboratory of Parasite Diseases, National Institutes of Health.

Sporozoite recovery. Sporozoites were obtained by gradient centrifugation. Using this procedure, large numbers of mosquitoes can be processed and the sporozoite preparation contains relatively small amounts of contaminants such as mosquito tissue, bacteria and fungi (6). Eight hundred to one thousand mosquitoes, with an infection rate of not less than 70% were used for each 10 ml gradient.

Thoracic hemocele sporozoites were recovered 15 to 18 days after the infective blood meal by isolation of mosquito thoraces. These parasite-containing mosquito tissues were suspended in cold tissue culture medium 199 (TC 199) and to each milliliter of this medium were added 200 units of penicillin and 200 g of streptomycin to minimize bacterial growth. To free the sporozoites, tissues were triturated in a loose fitting Teflon tissue grinder, and then centrifuged at 18 g for 5 minutes, to eliminate the heavier fragments of mosquito tissue. This sediment was discarded after being twice washed at the same speed, and the combined supernatants were centrifuged at 1320 g for 30 minutes. The parasites, now concentrated in the sediment, were re-suspended in 1 ml of TC 199 and layered on the surface of a 10 ml linear bovine serum albumin (BSA) renografin gradient. Details concerning the preparation of the gradient and the subsequent processing of the sporozoites have been published elsewhere (6).

Sporozoite irradiation and immunization schedule. Sporozoite suspensions of P. berghei were placed in serum vials and irradiated at 15 K rads in a Gammator (Radiation International, Inc.) by a ^{137}Cs source giving a uniform dose rate of 463 rads/min. P. cynomolgi-infected mosquitoes were exposed to 20 K rads of irradiation.

Mice were immunized by the i.v. injection of a primary dose of 7.5×10^4 irradiated P. berghei sporozoites followed at weekly intervals by 4 additional i.v. injections of 2.0×10^4 irradiated parasites. The rhesus whose serum was used in the present experiments, had been immunized by the repeated bites of irradiated P. cynomolgi-infected mosquitoes over a period of 16 months. This animal did not develop a sterile immunity but did show an extended prepatent period when challenged with viable P. cynomolgi sporozoites.

To obtain immune serum mice were bled from the retro-orbital venous plexus one week following the last immunizing dose. The rhesus was bled from the femoral vein. All sera were stored at -80°C prior to use.

Antisporozoite activity of immune sera. Antisera were selected following determination of their circumsporozoite precipitate (CSP) activity. The CSP titer, defined as the highest serum dilution producing a thread-like precipitate visible under phase microscopy at one end of the sporozoites, was determined according to a previously described technique (13). Mouse anti-P. berghei serum with a CSP titer of 1:40 and rhesus anti-P. cynomolgi serum with a CSP titer of 1:20 were used in the present experiments.

Immune serum incubation of sporozoites. Gradient recovered washed sporozoites of both P. berghei and P. cynomolgi were incubated within their respective immune sera for either 30 minutes at 37°C, 30 minutes at 4°C or 18 hours at 4°C. Control sporozoites, of P. berghei, and P. cynomolgi were incubated in their respective normal sera or TC 199 for 30 minutes at 37°C. Sporozoites of P. knowlesi were maintained only in TC 199. Following these procedures, the sporozoites preparations were three times washed in TC 199 at 4°C.

Electron microscopy. After washing, the parasites were fixed in 1.25% glutaraldehyde, 0.1 M cacodylate buffer, (pH 7.3), and 0.116 M sucrose. They were post fixed in 1% osmium tetroxide for 1 hour, dehydrated and then embedded in Epon 812. Sections were cut with a Porter-Blum MT-2 ultramicrotome with a Dupont diamond knife, mounted on copper grids and stained with 1% uranyl acetate and lead citrate. These sections were examined with a Siemens Elmiskop 101 electron microscope.

For freeze-fracturing, the glutaraldehyde-fixed samples were washed several

times in 0.1 M cacodylate buffer and soaked for several hours in the same buffer containing 20% glyceral as an antifreeze agent. Freeze-fracturing was performed according to the methods of Steere (11) in a Denton freeze-etching device. Fracturing was carried out at -100 C under vacuum of 10^{-7} torrs, and the surface obtained was replicated with carbon and platinum. Replicas were cleaned in 1% sodium hydrochlorite to remove adherent organic material and rinsed in distilled water. These replicas were examined with a Siemens Elmiskop 101 electron microscope.

Results

a) Thin section transmission electron microscopy.

Since the fine structure of plasmodial sporozoites by thin section transmission electron microscopy has been reported by several investigators (2), only a brief description of the parasite is presented here. The pellicle of the sporozoite is composed of a plasma membrane, a doubled inner membrane and a row of subpellicular microtubules (Fig. 1). The sporozoite's pellicle appears to be covered with a very thin surface coat of fibrillar material loosely surrounding the outer membrane. The apical end is a truncated cone-shaped projection demarcated by polar rings. Electron-dense rhoptries and micronemes are present in the anterior portion and ductules extend to the tip of the apical end. The nucleus is situated in the mid-portion and the posterior portion is occupied by mitochondria and occasional electron-dense inclusions.

Light microscopy reveals that antisera induce the formation of a thread-like precipitate, the "circum-sporozoite precipitate reaction (CSP reaction)" at one end of the parasite (13). Further studies, using both transmission

surrounding the outer membrane of sporozoites incubated in immune serum (Fig. 2) and that the thread-like precipitate (CSP) reaction is located at the posterior end of the sporozoite (2). The inner structure of these parasites appears to be relatively unaltered. The surface coat is absent or minimal on parasites incubated in normal serum.

b) Freeze fracture of the sporozoites of P. cynomolgi, P. knowlesi, and P. berghei treated with normal sera or medium 199.

The terminology used for this paper follows the nomenclature of Branton et al. (1) (Table 1). Both the distribution and size of intramembranous particles (IMP) on the P face of the outer plasma membrane of the sporozoites of P. cynomolgi, P. knowlesi, and P. berghei appear similar. There are numerous IMP on the P face of the outer membrane and they are evenly distributed (Figs. 3,4). No reticulate pattern as described by McLaren et al. (8) on the P face of the outer membrane of the P. knowlesi trophozoite is noted. Each IMP measures about 90 A. The E face of the outer plasma membrane shows fewer IMP (Figs. 6,7) as compared to the P face of the plasma membrane and they are unevenly distributed with occasional clumping. The E face of the intermediate pellicular membrane (Fig. 4) has few IMP and shows linear, slightly raised ridges along the long axis of the parasite. The P face of the intermediate pellicular membrane (Figs. 6,10) shows many IMP on the fractured surface, although their number is fewer than those on the P face of the plasma membrane. They are aligned along the long axis of the sporozoite with regular interline spacing. On the P face of the inner pellicular membrane (Fig. 5), the intramembranous particles are aligned in very distinct rows conforming to the long axis

of the cell. The E face of the inner pellicular membrane shows a few randomly distributed IMP.

c) Freeze fracture of the sporozoites of P. cynomolgi and P. berghei after treatment with antisera.

A prominent change seen in freeze fractured sporozoites after treatment with anti-sera is the appearance of an outer layer of particle aggregates surrounding the parasites (Figs. 9,10,11). The layer of these particle aggregates measures 200 nm in thickness and appears to correspond to the surface coat seen in thin section transmission electron microscopy.

The P face of the outer plasma membrane shows randomly distributed clear areas (Fig. 8) and aggregations of IMP (Fig. 9). These changes are not detected in sporozoites incubated with normal serum or TC 199. No changes are detectable in the other fractured faces of the pellicle. As is observed by the thin sectioning technique, deformed sporozoites with a large bulge in the midportion of the parasite are also noted (Fig. 11).

Discussion

Although there have been reports on freeze fracture studies of malarial parasites in the past (7), these studies dealt mainly with the structure of the intracellular organelles of the erythrocytic parasites. Recently McLaren et al. (8) using the freeze fracture technique described the pellicular membrane of erythrocytic P. knowlesi parasites as well as the membrane of the parasitophorous vacuole. The density of the IMP on each fracture surface of the pellicular membranes of the merozoite appears to be similar to that of the sporozoites reported in this current study. On the other hand, they failed to observe aligned IMP along the long axis on the P faces of the intermediate and inner

pellicular membranes. This observation may be due to differences in the preparation of the specimen.

Both the distribution and arrangement of the IMP in the pellicular membranes of the sporozoites of malarial parasites are similar to those of Eimerian sporozoites as reported by Dubremetz and Torpier (4). The aligned IMP along the long axis of the sporozoites appear to correspond to the underlying subpellicular microtubules. Similar arrangement of IMP is also described for Trypanosoma brucei (5), Sarcocystis tenella (10), and Toxoplasma gondii (10). However, the functional relationship between subpellicular microtubules and aligned IMP is not known.

Of particular interest is the similarity of the IMP distribution and arrangement on the P faces of both the intermediate and inner membranes. Also, the E face of the intermediate membrane and the E face of the inner membrane are similar in structure. This similarity may support a contention that the intermediate and inner membrane originated from flattened vesicles under the plasma membrane (4).

Freeze fracture replica of the sporozoites after treatment with antisera demonstrated the redistribution of intramembranous particles on the P face of the plasma membrane. On the other hand, the IMP of the intermediate and inner pellicular membranes did not show any detectable alteration in their distribution and number. Therefore, it appears that the immunoglobulins alter only the structural arrangement of the plasma membrane. Specific antibodies have been demonstrated to induce membrane surface reorganization. Douglas (3) reported similar IMP reorganizations on both the P and E faces of the plasma membrane of rabbit pulmonary alveolar macrophages after incubation with immunoprotein. Hogan and Patton (5) demonstrated aggregates

of IMP on the P and E faces of the plasma membrane of T. brucei which were obtained from intact rats, while parasites from immunosuppressed rats did not show such aggregates. They suggested that antigen-specific antibody brings about this aggregation. Our results, however, indicate IMP aggregates are only found on the P face of the plasma membrane.

The IMP-like particles surrounding sporozoites incubated in immune serum appear to correspond to the electron dense surface coat seen by the thin sectioning. The fact that this electron-dense surface coat has been demonstrated to be an immune complex by immuno-electron microscopy (2) suggests aggregates of the particles to be the immune complex. The aggregates of IMP on the P face of the plasma membrane and surface coat formation may be related phenomena. Reorganization of IMP in the plasma membrane may lead to the eventual formation of the surface coat.

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Diagram: Schematic diagram showing the fracture faces of a malarial sporozoite.

Figure Legends

Fig 1. Electron micrograph of a P. cynomolgi sporozoite incubated in T 199. The sporozoite surface is covered by a small amount of fibrillar material (arrow). 56,000X

Fig 2. Electron micrograph of P. cynomolgi sporozoite incubated in immune serum for 30 minutes at 37°C. The surface is covered by a prominent surface coat (arrow). 56,000X

Fig 3. Electron micrograph of a freeze fractured P. cynomolgi sporozoite. The P face (P_1) of the plasma membrane is uniformly covered by intramembranous particles. 61,000X

Fig 4. Electron micrograph of a freeze fractured P. cynomolgi sporozoite. IMP are numerous on the P face (P_1) of the plasma membrane, whereas they are fewer on the E face (E_2) of the intermediate membrane. 45,000X

Fig 5. Electron micrograph of a freeze fractured P. knowlesi sporozite showing IMP aligned to the long axis of the sporozoite on the P face (P_3) of the inner pellicular membrane. 60,000X

Fig 6. Electron micrograph of a freeze fractured P. knowlesi sporozoite showing the P (P_1) and E (E_1) faces of the plasma membrane, the P (P_2) and E (E_2) faces of the intermediate pellicular membrane and the P (P_3) face of the inner membrane. The P (P_1) face of the plasma membrane shows numerous IMP, while the E (E_1) face of the plasma membrane show fewer IMP. The E (E_2) face of the intermediate pellicular membrane shows hardly any IMP and the P (P_2) face shows few smaller

particles. The IMP on the P (P_3) face of the inner membrane appears to be aligned to the long axis of the sporozoite. 51,000X

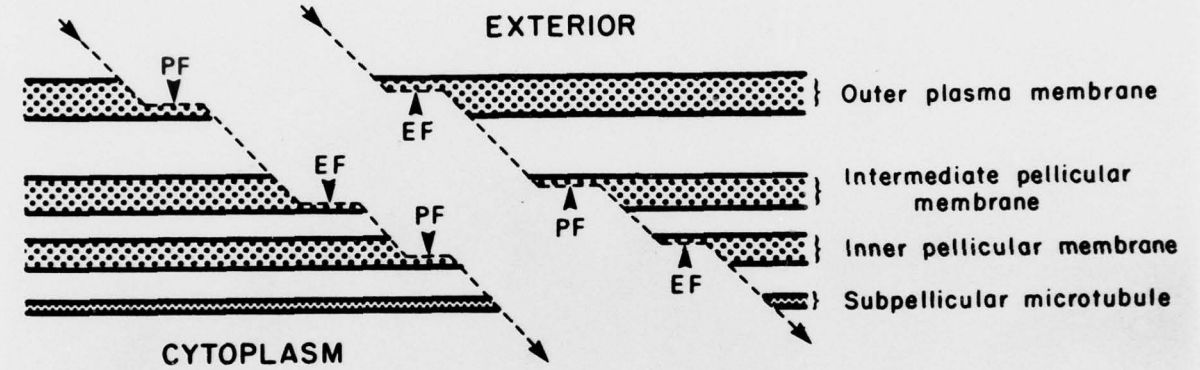
Fig 7. Electron micrograph of a freeze fractured P. berghei sporozoite showing fewer IMP on the E face (E_1) of the plasma membrane than on the P face (P_1) of the plasma membrane. 80,000X

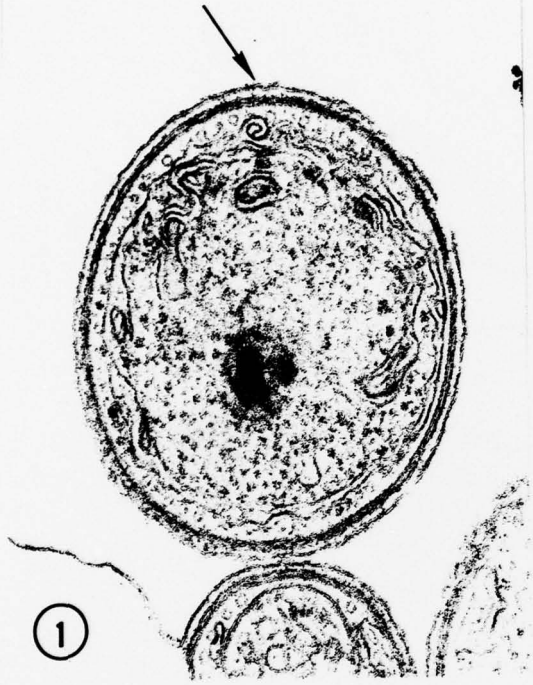
Fig 8. Electron micrograph of a freeze fractured P. cynomolgi sporozoite incubated in immune serum for 30 minutes at 37°C. IMP cover the P face (P_1) of the plasma membrane unevenly and the P face shows areas (arrow) without IMP. 45,000X

Fig 9. Electron micrograph of a freeze fractured P. cynomolgi sporozoite incubated in immune serum showing clumped IMP (arrow). The outline of the sporozoite is covered with a layer of particles (double arrow). 51,000X

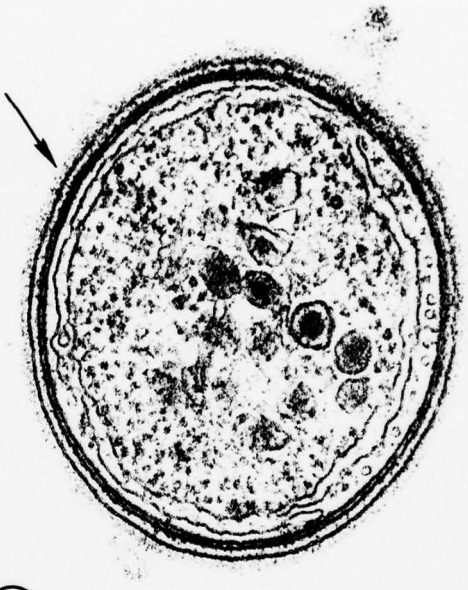
Fig 10. Freeze fractured P. cynomolgi sporozoite incubated in immune serum. The particles (arrow) along the outline of the sporozoite is seen. IMP on the E face (E_1) of the plasma membrane, P face (P_2) of the intermediate pellicular membrane and the E face (E_3) of the inner pellicular membrane appear not altered. The P face (P_2) of the intermediate membrane shows IMPs aligned along the long axis. 60,000X

Fig 11. Freeze fractured P. cynomolgi sporozoite incubated in immune serum. The sporozoite is deformed with a layer bulge in the midportion. Clumping of IMP on the P face (P_1) of the plasma membrane is evident. A layer of particles (arrow) cover the sporozoite surface (arrow). 47,000X


EXTERIOR
PF
EF
EF
PF
PF
EF
CYTOPLASM
Outer plasma membrane
Intermediate pellicular membrane
Inner pellicular membrane
Subpellicular microtubule



(1)



(2)

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4
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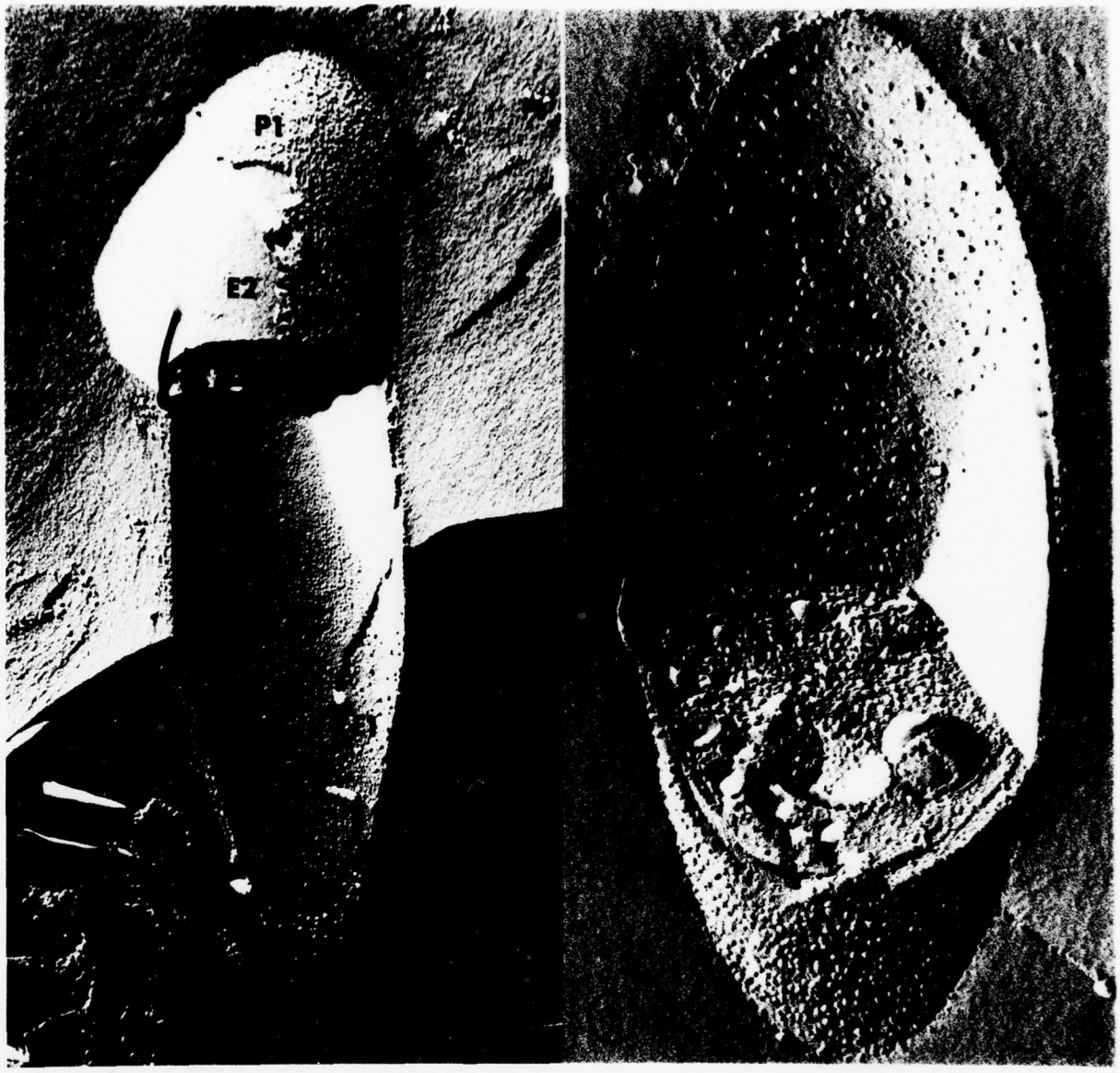
P1
EZ

9

11

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